

April 22, 2010

Attorney Docket No.: S2071-701019

Commissioner for Patents
MAIL STOP : HATCH-WAXMAN PTE
P.O. Box 1450
Alexandria, VA 22313-1450

Presented for filing is an Application for Extension of Patent Term for:

Inventors: Peter Francis Daniel

Patent No.: 7,138,262 B1

Issued: November 21, 2006

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING
HIGH MANNOSE PROTEINS

Assignee: Shire Human Genetic Therapies, Inc.

Enclosed are the following items, including those required for filing an Application For Extension of Patent Term under 35 U.S.C §. 156:

	<u>Pages</u>
Application for Extension of Patent Term	42
Attachment A Power of Attorney	6 (including cover page)
Attachment B Package insert for VPRIV™	3 (including cover page)
Attachment C NDA approval letter from FDA to Shire Human Genetic Therapies, Inc., dated February 26, 2010	19 (including cover page)
Attachment D U.S. Patent No.: 7,138,262 B1	30 (including cover page)
Attachment E Maintenance Fee Statement	2 (including cover page)
Attachment F Brumshtein et al. (2010) Glycobiology 20(1):24-32	10 (including cover page)
Attachment G Letter from FDA to Transkaryotic Therapies, Inc. dated 06/14/2010, providing the IND number and showing the date of receipt by FDA of the IND	4 (including cover page)
Attachment G1 A written record of the discussion that occurred on January 28, 2004 regarding modification of the protocol	2 (including cover page)
Attachment G2 Letter from Transkaryotic Therapies, Inc. to FDA dated March 11, 2004, concerning amendment of protocol	3 (including cover page)

Application for Extension of Patent Term
In re US Patent No.: 7,138,262 B1
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Express Mail Label No.: EB575684815US


Attachment G3	Communication from FDA to Transkaryotic Therapies, Inc. dated May 20, 2004, concerning amendment of protocol	3 (including cover page)
Attachment H	Letter from FDA to Shire Human Genetic Therapies, Inc. indicating the date IND was put on clinical hold	9 (including cover page)
Attachment I	Letter from FDA to Shire Human Genetic Therapies, Inc., dated December 21, 2006, removing the clinical hold and indicating that the protocol can be initiated	4 (including cover page)
Attachment J	Letter from FDA to Shire Human Genetic Therapies, Inc., dated September 14, 2009, acknowledging receipt of the final submission of the NDA	4 (including cover page)
Attachment K	Certification of Copies of Application Papers (x2 – 141 pgs each)	283 (including cover page)
Copy of this Transmittal Letter		2
Postcard listing items in submission (with number of pages for each)		NA
check in the amount of \$1,120.00		NA
2 additional copies of the Application as requested in MPEP 2753		2 x 141

If there are any questions regarding this filing, it is found to be incomplete, or if a telephone conference would otherwise be helpful, please call the undersigned at (617) 395-7000.

Kindly acknowledge receipt of this Application for Extension of Patent Term by returning the enclosed postcard.

Please direct all correspondence to the following:

37462
PTO Customer Number

Respectfully submitted,

Laurie Butler Lawrence
Reg. No. 46,593
Enclosures
LBL/sec

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Attorney Docket No.: S2071-701019

COPY

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Respectfully submitted,


Laurie Butler Lawrence

Reg. No. 46,593

Enclosures

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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PATENT EXTENSION
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CERTIFICATE OF EXPRESS MAILING UNDER 37 C.F.R. §1.10

The undersigned hereby certifies that this document was deposited with the U.S. Postal Service on April 22, 2010 for express mailing in accordance with §1.6(a)(2).


Laurie Butler Lawrence, Reg. No. 46,593

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Commissioner for Patents

P.O. Box 1450

Alexandria, VA 22313-1450

APPLICATION FOR EXTENSION OF PATENT TERM UNDER 35 U.S.C. § 156

Applicant, Shire Human Genetic Therapies, Inc. represents that it is the Assignee of the entire interest in and to United States Patent No. 7,138,262 B1 granted to Shire Human Genetic Therapies, Inc. on the 21st day of November 2006, for "High Mannose Proteins and Methods of Making High Mannose Proteins" by virtue of an assignment from Peter Francis Daniel to Transkaryotic Therapies, Inc., recorded in the U.S. Patent and Trademark Office at Reel 011662, Frame 0815, on March 28, 2001, and from Transkaryotic Therapies, Inc. to Shire Human Genetic Therapies, Inc., recorded in the U.S. Patent and Trademark Office at Reel 018224, Frame 0390, on August 31, 2006.

By the Power of Attorney enclosed herein (Attachment A), Applicant has appointed several individual attorneys, including Laurie Butler Lawrence, as attorneys for Shire Human Genetic Therapies, Inc. with regard to this application for extension of the term of U.S. Patent No. 7,138,262 B1 and to transact all business in the U.S. Patent and Trademark Office in connection therewith.

In re U.S. Patent No.: 7,138,262 B1
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Transkaryotic Therapies, Inc. became Shire Human Genetic Therapies, Inc. on January 17, 2006. Shire Human Genetic Therapies, Inc. is the holder of the regulatory approval granted with respect to the regulatory review period relied on herein.

Information Required Under 37 C.F.R. § 1.740

Applicant hereby submits this application for extension of the patent term under 35 U.S.C. § 156 by providing the following information required by the rules promulgated by the U.S. Patent and Trademark Office (37 C.F.R. § 1.740). For the convenience of the Patent and Trademark Office, the information contained in this application will be presented herein in a format which follows the order of the requirements of Section 1.740 of Title 37 of the Code of Federal Regulations.

(1) Identification of the Approved Product [1.740(a)(1)]

The approved product is VPRIVTM. The name of the active ingredient in VPRIVTM is velaglucerase alfa for injection. Velaglucerase alfa for injection is a hydrolytic lysosomal glucocerebroside-specific enzyme indicated for long-term replacement therapy (ERT) for pediatric and adult patients with type 1 Gaucher disease. The active ingredient of VPRIVTM is velaglucerase alfa, which is produced by gene activation technology in a human fibroblast cell line. Velaglucerase alfa is a glycoprotein of 497 amino acids; with a molecular weight of approximately 63 kDa. Velaglucerase alfa has the same amino acid sequence as the naturally occurring human enzyme, β -glucocerebrosidase. Velaglucerase alfa contains 5 potential N-linked glycosylation sites; four of these sites are occupied by glycan chains. Velaglucerase alfa is manufactured to contain predominantly high mannose-type N-linked glycan chains. VPRIVTM is supplied as a sterile, preservative free, lyophilized powder in single-use vials. Following reconstitution with Sterile Water for Injection, USP, the solution contains the components listed in Table 3 of the package insert, which is provided in Attachment B (a copy of the

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package insert is also provided as an enclosure to the NDA approval letter from FDA to Shire Human Genetic Therapies, Inc., dated February 26, 2010 in Attachment C).

(2) Federal Statute Governing Regulatory Approval of the Approved Product [1.740(a)(2)]

The approved product, VPRIVTM, was subject to regulatory review under § 505(i) and §505(b) of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. § 355(i) and § 355(b)).

(3) Date of Approval for Commercial Marketing [1.740(a)(3)]

The approved product, VPRIVTM, received permission for commercial marketing or use under Section 505(b) of the Federal Food, Drug, and Cosmetic Act on February 26, 2010. A copy of the NDA approval letter from FDA to Shire Human Genetic Therapies, Inc., dated February 26, 2010 (with enclosure), is provided as Attachment C.

(4) Identification of Active Ingredient and Certifications Related to Commercial Marketing of Approved Product [1.740(a)(4)]

The only active ingredient in VPRIVTM is velaglucerase alfa for injection which, on information and belief, has not been previously approved for commercial marketing or use under the Public Health Service Act, the Virus-Serum-Toxin Act or the Federal Food, Drug, and Cosmetic Act. A copy of the package insert describing the approved product is attached (Attachment B).

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**(5) Statement Regarding Timeliness of Submission of Patent Term
Extension Request [1.740(a)(5)]**

This application for extension of patent term under 35 U.S.C. § 156 is being submitted within the permitted 60-day period pursuant to 37 C.F.R. § 1.720(f). The last day on which this application can be submitted is April 26, 2010.

**(6) Complete Identification of the Patent for Which Extension Is Being
Sought [1.740(a)(6)]**

The complete identification of the patent for which a term extension is being sought is as follows:

Inventors:	Peter Francis Daniel
Patent No.:	7,138,262 B1
Filing Date:	August 18, 2000
Issue Date:	November 21, 2006
Expiration Date:	August 18, 2020

**(7) Copies of the Patent for Which an Extension is Being Sought
[1.740(a)(7)]**

A copy of U.S. Patent No.: 7,138,262 B1 is provided as Attachment D.

**(8) Copies of Disclaimers, Certificates of Correction, Receipt of
Maintenance Fee Payments, or Reexamination Certificate [1.740(a)(8)]**

(a) U.S. Patent No.: 7,138,262 B1 is not subject to a terminal disclaimer.

(b) No certificate of correction has been issued for U.S. Patent No.: 7,138,262 B1.

(c) The first maintenance fee for U.S. Patent No.: 7,138,262 B1 will be due with a payment of the surcharge on May 22, 2010. This maintenance fee has been paid as

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shown in the copy of the USPTO's on-line record of patent maintenance fee payment for this patent which is attached (Attachment E).

(d) U.S. Patent No.: 7,138,262 B1 has not been the subject of a reexamination proceeding.

(9) Statement Regarding Patent Claims Relative to Approved Product
[1.740(a)(9)]

The following claims of U.S. Patent No. 7,138,262 B1 claim a method of manufacturing the approved product, VPRIVTM: claims 1-4, 12-18, 23, 26-38, 48-54, and 57-61.

(iii) Pursuant to M.P.E.P. § 2753 and 37 C.F.R. § 1.740(a)(9)(iii), the following explanation is provided which demonstrates the manner in which at least one such patent claim reads on the method of manufacturing the approved product, VPRIVTM.

Description of the approved product and the method of manufacturing the same:

Velaglucerase alfa for injection is human β -glucocerebrosidase produced by gene-activation in immortalized human fibroblast HT-1080 cells. Gene activation refers to the introduction of an exogenous promoter into the cell that activates the endogenous human β -glucocerebrosidase gene. The activated gene expresses human β -glucocerebrosidase. β -glucocerebrosidase has 5 potential N-linked glycosylation sites, four of which are occupied by glycan chains in velaglucerase alfa for injection.

Glycosylation of velaglucerase alfa for injection is altered by culturing the cells in the presence of kifunensine, a mannosidase I inhibitor, at 2 μ g/ml. This results in the secretion of human β -glucocerebrosidase containing primarily high-mannose type glycan chains having 6-9 mannose units per glycan chain. The cells are cultured under conditions wherein:

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the mannosidase inhibitor prevents removal of one or more $\alpha 1,2$ mannose residue(s) distal to the pentasaccharide core; the removal of one or more mannose residues distal to the pentasaccharide core is prevented on at least two carbohydrate chains of hmGCB; at least 60% of the high mannose glucocerebrosidase (hmGCB) of the preparation have three or more carbohydrate chains in which the removal of one or more mannose residues distal to the pentasaccharide core has been prevented; at least about 60% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues; the mannosidase inhibitor further prevents the removal of one $\alpha 1,3$ mannose residue distal to the pentasaccharide core; the mannosidase inhibitor further prevents the removal of one $\alpha 1,6$ mannose residue distal to the pentasaccharide core; the mannosidase inhibitor prevents removal of at least three mannose residues distal to the pentasaccharide core of the precursor oligosaccharide of GCB; at least 60% of the hmGCB of the preparation have one or more carbohydrate chains in which the removal of three or more mannose residues distal to the pentasaccharide core has been prevented; at least 60% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues; and at least about 80% or more of the carbohydrate chains of the hmGCB preparation have six or more mannose residues.

Velaglycerase alfa for injection is harvested from media in which the cells are cultured.

Velaglycerase alfa for injection is secreted as a monomeric glycoprotein of approximately 63 kDa and is composed of 497 amino acids with a sequence identical to the natural human protein. The amino acid sequence of velaglycerase alfa for injection is described in Zimran et al. (2007) *Blood Cells Mol Dis*, 39: 115-118. Velaglycerase alfa for injection contains 5 potential N-linked glycosylation sites; four of these sites are occupied by glycan chains.

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A summary of the glycan structure, and other aspects of velaglucerase alfa for injection, is provided in Brumshtein et al. (2010) Glycobiology 20(1):24-32 as Attachment F. See, e.g., Table III, therein.

As is discussed below, claims 1-4, 12-18, 23, 26-38, 48-54, and 57-61 of U.S. Patent No. 7,138,262 B1 read on the method of manufacturing the approved product. The claims are set out in the left hand column of the table immediately below. The method of manufacturing the approved product is described in the right hand column and compared with the claim. As is shown, the approved product meets all of the limitations of each of claims 1-4, 12-18, 23, 26-38, 48-54, and 57-61 and claims 1-4, 12-18, 23, 26-38, 48-54, and 57-61 cover the method of manufacturing the approved product, VPRIV™.

<p>1. A method of producing a preparation of high mannose glucocerebrosidase (hmGCB) comprising a carbohydrate chain having at least four mannose residues,</p> <p>comprising: providing a mammalian cell that expresses a human glucocerebrosidase (GCB);</p>	<p>VPRIV™ includes at least two glucocerebrosidase (GCB) proteins that have at least one carbohydrate chain having four or more mannose residues. See, e.g., page 24, column 1 of Attachment F, “the predominant glycan on velaglucerase alfa is a high mannose type, with nine mannose residues”. See also Table III and Figures 7 and 8 of Attachment F. See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>VPRIV™ is produced in a human cell line that expresses human glucocerebrosidase See page 24, column 2 of Attachment F: “we have used gene activation in a well characterized, continuous human cell line to produce gene activated human acid-β-glucocerebrosidase (velaglucerase alfa).” See also the description of the approved product and the method of manufacturing</p>
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<p>contacting the cell with kifunensine; allowing the cell to produce hmGCB; and</p> <p>harvesting the hmGCB from the cell or its culture media, to thereby produce an hmGCB preparation.</p>	<p>the same in this section (9)(iii).</p> <p>The human cell line expressing VPRIVTM is contacted with kifunensine and the cells secrete VPRIV. See page 24, column 2 of Attachment F, "glycosylation of velaglycerase alfa is altered by using kifunensine ... during cell culture, which results in the secretion of a protein containing predominantly high-mannose type glycans" See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>VPRIVTM is harvested from the cell culture to produce a preparation with at least two glucocerebrosidase proteins having at least one carbohydrate chain having four or more mannose residues. See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 1 and the claim covers the method of manufacturing the approved product.</p>
<p>2. The method of claim 1, wherein removal of one or more α1,2 mannose residue(s) distal to the pentasaccharide core is prevented.</p>	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>VPRIVTM includes at least 2 GCB proteins that have one or more mannose residue distal to the pentasaccharide core present. See the description of the approved product and the method of manufacturing the same</p>

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	<p>in this section (9)(iii).</p> <p>Therefore, the method of making VPRIV meets all of the limitations of claim 2 and the claim covers the method of manufacturing the approved product.</p>
3. The method of claim 1, wherein the kifunensine is present at a concentration between about 0.05 to 20.0 µg/ml.	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>Kifunensine is present at a concentration 2.0 µg/ml in the method of making the approved product. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 3 and the claim covers the method of manufacturing the approved product.</p>
4. The method of claim 3, wherein the kifunensine is present at a concentration between about 0.1 to 2.0 µg/ml.	<p>As discussed above for claims 1 and 3, the method of making the approved product meets all of the limitations of the base claims.</p> <p>Kifunensine is present at a concentration of 2.0 µg/ml in the method of making the approved product. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM</p>

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	meets all of the limitations of claim 4 and the claim covers the method of making the approved product.
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12. The method of claim 1, wherein the removal of one or more mannose residues distal to the pentasaccharide core is prevented on at least two carbohydrate chains of hmGCB.	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>Kifunensine is an inhibitor of endoplasmic reticulum mannosidase I and Golgi mannosidase I enzymes (see column 23, lines 48-51 of the '262 patent) that prevents the removal of one or more mannose residues distal to the pentasaccharide core. (see, e.g., Fig. 7 of Attachment F which shows the predominant glycan structure present on the GCB in VPRIVTM). See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 12 and the claim covers the method of manufacturing the approved product.</p>
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13. The method of claim 1, wherein at least 60% of the hmGCB of the preparation have one or more carbohydrate chains in which the removal of one or more mannose residues distal to the pentasaccharide core has been prevented.	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>At least 60% of the hmGCB of the preparation have one or more carbohydrate chains in which the removal of one or more</p>
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	<p>mannose residues distal to the pentasaccharide core has been prevented. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 13 and the claim covers the method of manufacturing the approved product.</p>
14. The method of claim 13, wherein the removal of three or more mannose residues distal to the pentasaccharide core has been prevented.	<p>As discussed above for claims 1 and 13, the method of making the approved product meets all of the limitations of the base claims.</p> <p>The removal of three or more mannose residues distal to the pentasaccharide core has been prevented. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 14 and the claim covers the method of manufacturing the approved product.</p>
15. The method of claim 1, wherein at least about 20% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>At least about 20% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues. See the description of the</p>

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	<p>approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 15 and the claim covers the method of manufacturing the approved product.</p>
<p>16. The method of claim 15, wherein at least about 40% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.</p>	<p>As discussed above for claims 1 and 15, the method of making the approved product meets all of the limitations of the base claim.</p> <p>At least about 40% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 16 and the claim covers the method of manufacturing the approved product.</p>

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<p>17. The method of claim 16, wherein at least about 60% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.</p>	<p>As discussed above for claims 1, 15 and 16, the method of making the approved product meets all of the limitations of the base claims.</p> <p>At least about 60% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 17 and the claim covers the method of manufacturing the approved product. .</p>
<p>18. The method of claim 1, wherein at least about 80% or more of the carbohydrate chains of the hmGCB preparation have six or more mannose residues.</p>	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>At least about 80% or more of the carbohydrate chains of the hmGCB preparation have six or more mannose residues. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 18 and the claim covers the method of manufacturing the approved product.</p>

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<p>23. The method of claim 1, wherein the cell comprises an exogenous regulatory sequence which functions to regulate expression of an endogenous GCB coding sequence.</p>	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>VPRIVTM is produced by a human cell line that comprises an exogenous promoter sequence to regulate expression of an endogenous GCB coding sequence. (see, e.g., page 24, column 2 of Attachment F- “we have used gene activation in a well-characterized, continuous human cell line to produce gene activated human acid-β-glucocerebrosidase (velaglucerase alfa). Gene activation refers to targeted recombination with a promoter that activates the endogenous GlcCerase gene in the selected human cell line.” See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 23 and the claim covers the method of manufacturing the approved product.</p>
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<p>26. The method of claim 1, wherein the cell is a human cell.</p>	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>VPRIVTM is produced by a human cell line. See page 24, column 2 of Attachment F – “we have used gene activation in a well characterized, continuous human cell line to produce gene activated human acid-β-glucocerebrosidase (velaglucerase alfa).” See also the description of the approved product and the method of manufacturing</p>
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	<p>the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 26 and the claim covers the method of manufacturing the approved product.</p>
27. The method of claim 26, wherein the cell is a fibroblast or a myoblast.	<p>As discussed above for claims 1 and 26, the method of making the approved product meets all of the limitations of the base claims.</p> <p>VPRIVTM is produced by HT-1080 cells which are fibroblasts. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 27 and the claim covers the method of manufacturing the approved product.</p>
28. The method of claim 26, wherein the cell is an immortalized cell.	<p>As discussed above for claims 1, 26 and 27, the method of making the approved product meets all of the limitations of the base claims.</p> <p>VPRIVTM is produced by an immortalized cell. See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 28 and the claim covers the method of making the</p>

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	approved product.
29. The method of claim 27, wherein the cell is an HT-1080 cell.	<p>As discussed above for claims 1, 26, 27 and 28, the method of making the approved product meets all of the limitations of the base claims.</p> <p>VPRIVTM is produced by HT-1080 cells. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 29 and the claim covers the method of manufacturing the approved product.</p>
30. The method of claim 1, wherein the cell is contacted with kifunensine in culture media.	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>VPRIVTM is produced by contacting the cells expressing hGCB with kifunensine in cell culture. See page 24, column 2 of Attachment F, "glycosylation of velaglucerase alfa is altered by using kifunensine ... during cell culture, which results in the secretion of a protein containing predominantly high-mannose type glycans." See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 30 and the claim covers the method of manufacturing the approved product.</p>

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31. The method of claim 30, wherein the hmGCB is obtained from the media in which the cell is cultured.	<p>As discussed above for claims 1 and 30, the method of making the approved product meets all of the limitations of the base claims.</p> <p>VPRIVTM is obtained from the media in which the human cell line is cultured. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 31 and the claim covers the method of manufacturing the approved product.</p>

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<p>32. A method of producing a preparation of high mannose glucocerebrosidase (hmGCB) comprising a carbohydrate chain having at least four mannose residues, the method comprising:</p> <p>providing a human cell into which a nucleic acid sequence comprising an exogenous regulatory sequence has been introduced such that the regulatory sequence is operably linked to, and regulates the expression of, an endogenous GCB coding region;</p> <p>contacting the cell with a class 1 mannosidase inhibitor such that the removal of at least one mannose residue distal to the pentasaccharide core of a precursor oligosaccharide of GCB is prevented; and allowing the cell to produce hmGCB, to thereby produce an hmGCB preparation.</p>	<p>VPRIVTM includes at least two glucocerebrosidase (GCB) proteins having at least one carbohydrate chain having four or more mannose residues. See, e.g., page 24, column 1 of Attachment F, “the predominant glycan on velaglycerase alfa is a high mannose type, with nine mannose residues”. See also Table III and Figures 7 and 8 of Attachment F. See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>VPRIVTM is produced by a human cell that includes an exogenous promoter sequence to regulate expression of an endogenous GCB coding sequence. (see, e.g., page 24, column 2 of Attachment F-“we have used gene activation in a well-characterized, continuous human cell line to produce gene activated human acid-β-glucocerebrosidase (velaglycerase alfa). Gene activation refers to targeted recombination with a promoter that activates the endogenous GlcCerase gene in the selected human cell line.” See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>VPRIVTM is produced by contacting the cells expressing hGCB with kifunensine, which is a class 1 mannosidase inhibitor, in cell culture. See page 24, column 2 of Attachment F, “glycosylation of velaglycerase alfa is altered by using kifunensine ... during cell culture, which results in the secretion of a protein</p>
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	<p>containing predominantly high-mannose type glycans". VPRIVTM includes at least 2 GCB proteins having at least one carbohydrate chain with at least one mannose residue distal to the pentasaccharide core, e.g., the GCB proteins have four or more mannose residues. See, e.g., Table III of Attachment F. See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 32 and the claim covers the method of manufacturing the approved product.</p>
33. The method of claim 32, wherein the mannosidase inhibitor prevents the removal of one or more α 1,2 mannose residue(s) distal to the pentasaccharide core.	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>The mannosidase inhibitor prevents the removal of one or more α1,2 mannose residue(s) distal to the pentasaccharide core. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 33 and the claim covers the method of manufacturing the approved product.</p>
34. The method of claim 32, wherein the mannosidase inhibitor further prevents the	As discussed above for claim 32, the method of making the approved product

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removal of one α 1,3 mannose residue distal to the pentasaccharide core.	<p>meets all of the limitations of the base claim.</p> <p>The mannosidase inhibitor further prevents the removal of one α1,3 mannose residue distal to the pentasaccharide core. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 34 and the claim covers the method of manufacturing the approved product.</p>
35. The method of claim 32, wherein the mannosidase inhibitor further prevents the removal of one α 1,6 mannose residue distal to the pentasaccharide core.	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>The mannosidase inhibitor further prevents the removal of one α1,6 mannose residue distal to the pentasaccharide core. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 35 and the claim covers the method of manufacturing the approved product.</p>
36. The method of claim 32, wherein the class 1 processing mannosidase inhibitor is kifunensine.	As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.

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	<p>VPRIVTM is produced by contacting the cells expressing hGCB with kifunensine in cell culture. See page 24, column 2 of Attachment F, "glycosylation of velaglycerase alfa is altered by using kifunensine ... during cell culture, which results in the secretion of a protein containing predominantly high-mannose type glycans". See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 36 and the claim covers the method of manufacturing the approved product.</p>
37. The method of claim 36, wherein the kifunensine is present at a concentration between about 0.05 to 20.0 µg/ml.	<p>As discussed above for claims 32 and 36, the method of making the approved product meets all of the limitations of the base claims.</p> <p>Kifunensine is present at a concentration 2.0 µg/ml in the method of making the approved product. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 37 and the claim covers the method of manufacturing the approved product.</p>

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<p>38. The method of claim 37, wherein the kifunensine is present at a concentration between about 0.1 to 2.0 µg/ml.</p>	<p>As discussed above for claims 32, 36 and 37, the method of making the approved product meets all of the limitations of the base claims.</p> <p>Kifunensine is present at a concentration 2.0 µg/ml in the method of making the approved product. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 38 and the claim covers the method of manufacturing the approved product.</p>
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<p>48. The method of claim 32, wherein the mannosidase inhibitor prevents removal of at least three mannose residues distal to the pentasaccharide core of the precursor oligosaccharide of GCB.</p>	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>Kifunensine is an inhibitor of endoplasmic reticulum mannosidase I and Golgi mannosidase I enzymes (see column 23, lines 48-51 of the '262 patent) that prevents the removal of three or more mannose residues distal to the pentasaccharide core. (see, e.g., Fig. 7 of Attachment F which shows the predominant glycan structure present on the GCB in VPRIVTM). See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p>
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	Therefore, the method of making VPRIV TM meets all of the limitations of claim 48 and the claim covers the method of manufacturing the approved product.
49. The method of claim 32, wherein the mannosidase inhibitor prevents removal of one or more mannose residues distal to the pentasaccharide core on at least two of the carbohydrate chains of hmGCB.	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>Kifunensine is an inhibitor of endoplasmic reticulum mannosidase I and Golgi mannosidase I enzymes (see column 23, lines 48-51 of the '262 patent) that prevents the removal of one or more mannose residues distal to the pentasaccharide core. (see, e.g., Fig. 7 of Attachment F which shows the predominant glycan structure present on the GCB in VPRIVTM). See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 49 and the claim covers the method of manufacturing the approved product.</p>
50. The method of claim 32, wherein at least 60% of the hmGCB of the preparation have one or more carbohydrate chains in which the removal of three or more mannose residues distal to the pentasaccharide core has been prevented.	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>At least 60% of the hmGCB of the preparation have one or more carbohydrate</p>

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	<p>chains in which the removal of three or more mannose residues distal to the pentasaccharide core has been prevented. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 50 and the claim covers the method of manufacturing the approved product.</p>
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51. The method of claim 32, wherein at least 20% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>At least 20% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 51 and the claim covers the method of manufacturing the approved product.</p>
52. The method of claim 51, wherein at least 40% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.	As discussed above for claims 32 and 51, the method of making the approved product meets all of the limitations of the base claims.

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	<p>At least 40% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 52 and the claim covers the method of manufacturing the approved product.</p>
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53. The method of claim 52, wherein at least 60% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.	<p>As discussed above for claims 32, 51 and 52, the method of making the approved product meets all of the limitations of the base claims.</p> <p>At least 60% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 53 and the claim covers the method of manufacturing the approved product.</p>
54. The method of claim 32, wherein at least about 80% or more of the carbohydrate chains of the hmGCB preparation have six or more mannose residues.	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>At least about 80% or more of the</p>

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	<p>carbohydrate chains of the hmGCB preparation have six or more mannose residues. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 54 and the claim covers the method of manufacturing the approved product.</p>
57. The method of claim 32, wherein the cell is a fibroblast or a myoblast.	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>VPRIVTM is produced by an HT-1080 cell line, which is a fibroblast. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 57 and the claim covers the method of manufacturing the approved product.</p>
58. The method of claim 32, wherein the cell is an immortalized cell.	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>VPRIVTM is produced by an immortalized cell. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p>

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	Therefore, the method of making VPRIV TM meets all of the limitations of claim 58 and the claim covers the method of making the approved product.
59. The method of claim 58, wherein the cell is an HT-1080 cell.	<p>As discussed above for claims 32 and 58, the method of making the approved product meets all of the limitations of the base claims.</p> <p>VPRIVTM is produced by an HT-1080 cell line. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 59 and the claim covers the method of manufacturing the approved product.</p>

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<p>60. The method of claim 36, wherein the cell is contacted with kifunensine in culture media.</p>	<p>As discussed above for claims 32 and 36, the method of making the approved product meets all of the limitations of the base claims.</p> <p>VPRIVTM is produced by contacting the cells expressing hGCB with kifunensine in cell culture. See page 24, column 2 of Attachment F, "glycosylation of velaglucerase alfa is altered by using kifunensine ... during cell culture, which results in the secretion of a protein containing predominantly high-mannose type glycans" See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 60 and the claim covers the method of manufacturing the approved product.</p>
<p>61. The method of claim 60, wherein the hmGCB is obtained from the media in which the cell is cultured.</p>	<p>As discussed above for claims 32, 36, and 60, the method of making the approved product meets all of the limitations of the base claims.</p> <p>VPRIVTM is obtained from the media that the cell line is cultured. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 61 and the claim covers the method of</p>

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	manufacturing the approved product.
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**(10) Relevant Dates Under 35 U.S.C. § 156 for Determination of
Applicable Regulatory Review Period [1.740(a)(10)]**

The relevant dates and information pursuant to 35 U.S.C. § 156(g) to enable the Secretary of Health and Human Services to determine the applicable regulatory review period are as follows:

Patent Issue Date:

U.S. Patent No. 7,138,262 B1 issued on November 21, 2006.

***(i)(A) IND Effective Date and IND number [35 U.S.C. §156(g)(1)(B)(i); 37
C.F.R. §1.740(a)(10)(i)(A)]***

The effective date of IND 61,220 was May 20, 2004.

An IND was by submitted by Transkaryotic Therapies, Inc. to FDA and received by FDA on December 31, 2003. It was assigned number IND 61,220. A copy of the letter from FDA to Transkaryotic Therapies, Inc., dated January 12, 2004, providing the IND number and showing the date of receipt by FDA of the IND is provided in Attachment G. On January 28, 2004, FDA notified Transkaryotic Therapies, Inc. that a modification to the protocol was necessary. A written record of the discussion is provided in Attachment G1. Transkaryotic Therapies, Inc submitted an amendment to the protocol on March 11, 2004, see letter from Transkaryotic Therapies, Inc. to FDA, dated March 11, 2004, concerning amendment of protocol, provided in Attachment G2. On May 20, 2004, FDA notified Transkaryotic Therapies, Inc. that it could proceed, see FDA communication to Transkaryotic Therapies, Inc. dated May 20, 2004, concerning amendment to protocol, a copy of which is provided in Attachment G3.

On November 20, 2006, FDA notified Shire Human Genetics Therapies, Inc. that IND 61,220 was on clinical hold. A copy of the letter from the FDA to Shire Human Genetics Therapies, Inc. showing the date the FDA notified Shire Human Genetics

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Therapies, Inc. of the clinical hold is provided as Attachment H. The FDA removed the clinical hold on December 21, 2006. A copy of the letter dated December 21, 2006 from the FDA to Shire Human Genetics Therapies, Inc. indicating that the hold had been removed and the protocol could be initiated is provided as Attachment I.

Thus, as set out above, the date that an exemption under §505(i) of the Federal Food, Drug and Cosmetic Act became effective (i.e., the date that an investigational new drug application (IND) became effective for VPRIVTM) was May 20, 2004.

(i)(B) NDA Submission Date [35 U.S.C. §156(g)(1)(B)(i); 37 C.F.R.

§1.740(a)(10)(i)(B)] The NDA was submitted on a rolling basis. The initial portion of the NDA was submitted by Shire Human Genetic Therapies, Inc. to the FDA on July 30, 2009. The final portion was submitted on August 31, 2009. This date is used in the calculations provided herein. The NDA was assigned number NDA 22575. A copy of a letter from FDA to Shire Human Genetic Therapies, Inc., dated September 14, 2009, acknowledging receipt of the final submission of the NDA application is provided as Attachment J.

(i)(C) NDA Approval Date [35 U.S.C. §156(g)(1)(B)(ii); 37 C.F.R.

§1.740(a)(10)(i)(C)]

The FDA approved NDA 22575 authorizing the marketing of VPRIVTM on February 26, 2010. VPRIVTM was approved under the Department of Health and Human Services (DHHS). A copy of the approval letter from FDA to Shire Human Genetic Therapies, Inc., dated February 26, 2009 is provided as Attachment C.

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A brief description of the significant activities undertaken by the marketing applicant during the applicable regulatory review period with respect to VPRIVTM and the dates applicable to these significant activities are set forth in a chronology of events provided below.

Date	IND or NDA Serial No. (if applicable)	Other Contact	Description
06 April 2001			Pre-IND Teleconference Request
18 Nov. 2003			Pre-IND Meeting
30 Dec. 2003	IND 61,220 Serial 000		Submission of Original IND (including general information on Gaucher disease, nonclinical data, manufacturing info., and Phase I/II study - TKT025 New Protocol and IB).
19 Jan. 2004		FDA letter	FDA Correspondence: Acknowledgement of Receipt of IND and assignment of IND number
28 Jan 2004		TCR Contact Report	FDA Medical Officer request protocol amendment as discussed at the Pre-IND meeting.
28 Jan 2004	IND 61,220 Serial 001		Response to email dated 13 Jan. 2004 containing questions on clarification of age, inclusion criteria, and genotyping.
11 Mar. 2004	IND 61,220 Serial 002		Protocol Amendment: Amendment 2 of Clinical Protocol TKT025
07 April 2004	IND 61,220 Serial 003		Protocol Amendment: New Investigator for TKT025 and Blinding procedures used in TKT025 .
20 May 2004		TCR Contact Report	FDA Medical Officer says it's safe to proceed with the blinding procedure as amended in Serial 003 dated 07 April 2004 for Study TKT025 .
25 Aug. 2004		FDA letter	Re: Completion of IND Preclinical Pharm/Tox review and comments/recommendations
02 Nov. 2004	IND 61,220 Serial 007		Protocol Amendment: TKT025 Protocol Amendment 4 and New Protocol TKT025EXT
24 Nov. 2004	IND 61,220 Serial 008		Information Amendment: Comparability Protocol – comprehensive plan for evaluating changes to manufacturing process for drug substance (switch from 3x 30L Bioreactor to 100 L Bioreactor).
06 Apr.	IND 61,220 Serial		Information Amendment: Pharm/Tox: Final Study

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Date	IND or NDA Serial No. (if applicable)	Other Contact	Description
2005	017		Reports: Rat & Monkey 6-Month tox studies.
04 Nov. 2005	IND 61,220 Serial 023		Type B Meeting (End of Phase II) Request
12 Dec. 2005	IND 61,220 Serial 024		End of Phase II Briefing Document
09 Jan. 2006		FDA Fax	FDA responses to EOP II Meeting questions
11 Jan. 2006			End of Phase II Meeting
07 Feb. 2006		FDA letter	Official FDA Minutes from EOPII meeting 11 Jan. 2006
30 Mar. 2006	IND 61,220 Serial 029		Information Amendment: CMC: Manufacturing process modifications: switch from 3x 30L to 500 L scale up (AF1 process).
12 April 2006	IND 61,220 Serial 030		Information Amendment: Pharm/Tox: supportive information to justify Nonclinical Development Program and request for teleconference
02 May 2006	IND 61,220 Serial 031		Type A Meeting Request to discuss adequacy of nonclinical development program to initiate P3 studies and support a NDA.
18 May 2006	IND 61,220 Serial 032		Type C Meeting on 16 June 2006: Pharm/Tox Briefing Package
15 June 2006		FDA Fax	FDA Correspondence: 16 June 2006 teleconference not necessary, based on FDA'S initial responses to questions.
23 June 2006		FDA letter	FDA Correspondence: Comments and request for additional information, re: amendment dated 12 April 2006, IND Serial 030.
12 July 2006		FDA letter	FDA Correspondence: Acknowledgement of Shire's decision to accept FDA's written responses in lieu of meeting.
28 July 2006	IND 61,220 Serial 034		Information Amendment: Pharm/Tox: Responses to FDA comments and requests to 23 June fax, rat and rabbit studies.
03 Aug. 2006	IND 61,220 Serial 035		Information Amendment: CMC: Description and comparability data of AF1 process material
22 Sept. 2006	IND 61,220 Serial 037		Protocol Amendment: New Protocol , Phase 2/3 Clinical Protocol TKT032
16 Nov. 2006	IND 61,220 Serial 039		Protocol Amendment: New Protocol , Phase 3 Clinical Protocol TKT034
20 Nov. 2006		TCR Contact Report	Teleconference: IND put on clinical hold over concerns about product comparability.
28 Nov.		FDA Fax	FDA Correspondence: Full Clinical Hold Letter

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Assignee: Shire Human Genetic Therapies,
Inc.Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Date	IND or NDA Serial No. (if applicable)	Other Contact	Description
2006			
28 Nov. 2006	IND 61,220 Serial 040		Type A Meeting Request, response to Clinical Hold.
29 Nov. 2006		TCR Contact Report	Teleconference: response to FDA concerns relating to DS comparability resulting in Full Clinical Hold.
30 Nov. 2006	IND 61,220 Serial 041		Complete Response to Full Clinical Hold Letter relating to DS comparability.
30 Nov. 2006	IND 61,220 Serial 042		Request for Partial Waiver of the Full Clinical Hold.
01 Dec. 2006		TCR Contact Report	Teleconference: FDA agrees to move the Full Clinical Hold to a partial hold after reviewed Shire's response.
07 Dec. 2006		FDA letter	FDA Correspondence: Partial Clinical Hold Letter
12 Dec. 2006	IND 61,220 Serial 043		Response to Clinical/Statistical non-hold issues raised in full Clinical Hold Letter (Study TKT032)
14 Dec. 2006	IND 61,220 Serial 044		Protocol Amendment for Study TKT025EXT.
21 Dec. 2006		FDA letter	FDA Correspondence: Removal of Partial Clinical Hold Note: all clinical issues have been resolved.
26 Feb. 2007	IND 61,220 Serial 047		Protocol Amendment: New Protocol HGT-GCB-039 and New Investigator for TKT032
04 Sept. 2007		FDA letter	FDA Correspondence: Request for Information-Study TKT034
24 Sept. 2007	IND 61,220 Serial 055		Response to FDA request for Information-Study TKT032
10 Dec. 2007	IND 61,220 Serial 057		Response to FDA Request for Information-Study TKT034
11 Dec. 2007	IND 61,220 Serial 058		Information Amendment: Comparability Protocol for drug substance cell culture scale-up (AF2) vs. AF1 process
19 Dec 2007	IND 61,220 Serial 060		Protocol Amendment: New Protocol HGT-GCB-044 (Extension study for TKT032, TKT034 and HGT- GCB-039).
03 Sept. 2008	IND 61,220 Serial 070		Information Amendment: Description and comparability data of AF2 process material
06 Oct. 2008	IND 61,220 Serial 072		Information Amendment: Description and comparability data of 200 U/vial presentation
30 April 2009			Request for Orphan Drug Designation to FDA OOPD
08 June 2009	IND 61,220 Serial 081		Type B Meeting Request: Pre-NDA Meeting
08 June 2009		FDA OOPD	FDA OOPD Correspondence: Orphan Drug Designation Granted, US ODD #09-2835

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
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Date	IND or NDA Serial No. (if applicable)	Other Contact	Description
		letter	
30 June 2009	IND 61,220 Serial 082		Submission of Treatment Protocol HGT-GCB-058
30 June 2009	IND 61,220 Serial 083		Request for Fast Track Designation
01 July 2009		FDA letter	FDA Correspondence: Type B Pre-NDA meeting Granted on 10 August, 2009
08 July 2009		FDA letter	FDA Correspondence: Acknowledgement of Fast Track designation Request
15 July 2009		FDA letter	FDA Correspondence: Fast Track Designation Granted
23 July 2009		TCR Contact Report	Plans for rolling NDA for velaglycerase alfa
27 July 2009	IND 61,220 Serial 088		Request for Submission of Portions of an NDA Application
29 July 2009		WHO Collaboratin g Centre for Drug Statistics Methodology	ATC application for velaglycerase alfa (Ref: 09/1527- 2/EPLI/TUGR). ATC Proposed Code: A16AB10 velaglycerase alfa.
30 July 2009		FDA letter	FDA Correspondence: Acknowledgement to Proceed with Treatment Protocol HGT-GCB-058
30 July 2009	NDA 022575, Sequence 0000		Submission of 1st wave Rolling NDA, including M3 (complete), M4 (complete), M5 (partial). And request for Priority Review of NDA
08 Aug. 2009		FDA Fax	FDA Correspondence: FDA preliminary response for Pre- NDA Meeting Briefing Package
10 Aug. 2009			Pre-NDA Meeting
31 Aug. 2009	NDA 022575, Sequence 0001		Submission of 2nd wave of Rolling NDA, including M1, M2 (complete), M3 (update), M5 (complete).
14 Sept. 2009		FDA email, fax, letter	FDA Correspondence: FDA Acknowledgement Letter of NDA Submission
22 Sept. 2009	NDA 022575, Sequence 0003		Request for Proprietary Name Review
01 Oct. 2009	NDA 022575, Sequence 0005		Trade Name Request –Labeling Supplement
30 Oct. 2009		FDA letter	FDA Correspondence: Filing Communication – Priority Review Granted, and a list of review questions included.
03 Nov. 2009	NDA 022575, Sequence 0011		Location of data to support Orphan Drug Designation
19 Nov.		FDA Fax	Request Clinical Information

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Date	IND or NDA Serial No. (if applicable)	Other Contact	Description
2009			
20 Nov. 2009		FDA Email String	FDA Inspection Announcement Letters - 2 sites Inspections (Dec 6-10, 14-18, 2009)
20 Nov. 2009	NDA 022575, Sequence 0012		Partial Response to Request for Information: FDA Questions dated 30 Oct. 2009
01 Dec. 2009	NDA 022575, Sequence 0013		3 Month Safety Update
04 Dec. 2009		Email String	FDA BIMO Visit to Shire HGT LTP Site (Dec 08, 2009)
04 Dec. 2009	NDA 022575, Sequence 0014		Complete Response to Request for Information: FDA Questions dated 30 Oct. 2009 and 19 Nov. 2009
11 Dec. 2009		FDA Fax	Request CMC, Clinical Information
16 Dec. 2009		FDA letter	Proprietary name request : Conditional acceptance
18 Dec. 2009	NDA 022575, Sequence 0016		Response to Request for Information: FDA letter dated 11 Dec. 2009 (CMC)
22 Dec. 2009	NDA 022575, Sequence 0017		Stability update (drug substance and drug product)
31 Dec. 2009	NDA 022575, Sequence 0018		Response to Request for Information: Quality and Efficacy
13 Jan. 2010	NDA 022575, Sequence 0019		Response to Request for Information: CMC Questions of 23 Dec. 2009 and 07 Jan 2010 letters
14 Jan. 2010	NDA 022575, Sequence 0020		Response to to Request for Information: Clinical Questions of 23 Dec. 2009 Fax
15 Jan. 2010	NDA 022575, Sequence 0021		Response to telephone request of 15 Jan. 2010 – CMC information
26 Jan. 2010	NDA 022575, Sequence 0022		Response to Request for Information regarding inspections: Responses to FDA Form 483 in Paraguay, Israel, Shire HGT (300 PW)
27 Jan. 2010	NDA 022575, Sequence 0023		Response to 22 Jan. 2010 FDA Request for CMC Information
29 Jan. 2010		FDA email & letter	FDA comments on US PI
01 Feb. 2010	NDA 022575, Sequence 0024		Response to 27 Jan. 2010 FDA Request for Clinical Information
08 Feb. 2010	NDA 022575, Sequence 0025		Response to 03 Feb. 2010 FDA Fax Request for Clinical Information
09 Feb. 2010	NDA 022575, Sequence 0026		Response to FDA labeling question dated 29 Jan. 2010
10 Feb. 2010		FDA email &	FDA comments on labeling-carton labeling and container labels

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Date	IND or NDA Serial No. (if applicable)	Other Contact	Description
		letter	
17 Feb. 2010		FDA email	Response to FDA fax 10 Feb 2010 on labeling-carton label and vial labels
17 Feb. 2010	NDA 022575, Sequence 0027		Response to FDA labeling question dated 10 Feb. 2010 (carton and container comments)
18 Feb. 2010		FDA Fax	FDA comments on labeling-carton and container labels
19 Feb. 2010	NDA 022575, Sequence 0028		Response to FDA labeling comments 17 Feb 2010 and carton and container label comments 18 Feb 2010
25 Feb. 2010	NDA 022575, Sequence 0029		Information Amendment: Final Post-Marketing Commitments and Final Labeling Text (Company agreed PMCs and labeling text)
25 Feb. 2010	NDA 022575, Sequence 0030		Information Amendment: Post-Marketing Commitments and Draft Labeling Text
26 Feb. 2010		FDA Action Letter	NDA Approval Letter

Issued: November 21, 2006

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Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
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(12) Statement Concerning Eligibility for and Duration of Extension

Sought Under 35 U.S.C. § 156 [37 C.F.R. §1.740(a)(12)]

(i) Applicant is of the opinion that U.S. Patent No. 7,138,262 B1 is eligible for extension of the patent term under 35 U.S.C. § 156 of 687 days and should be extended until July 6, 2022. It satisfies all requirements for such extension including:

(a) 35 U.S.C. § 156(a) - U.S. Patent No. 7,138,262 B1 claims a method of manufacturing the approved product, VPRIVTM.

(b) 35 U.S.C. § 156(a)(1) - U.S. Patent No. 7,138,262 B1 has not expired before submission of this application.

(c) 35 U.S.C. § 156(a)(2) - The term of U.S. Patent No. 7,138,262 B1 has never been extended under 35 U.S.C. § 156(e)(1).

(d) 35 U.S.C. § 156(a)(3) - The application for patent term extension is submitted by the owner of record of the patent in accordance with the requirements of paragraphs (1) through (4) of 35 U.S.C. § 156(d) and the rules of the Patent and Trademark Office.

(e) 35 U.S.C. § 156(a)(4) - The product VPRIVTM has been subject to a regulatory review period before its commercial marketing or use.

(f) 35 U.S.C. § 156(a)(5)(A) - The commercial marketing or use of the product VPRIVTM after the regulatory review period is the first permitted commercial marketing or use under the provisions of § 505(b) of the Federal Food, Drug, and Cosmetic Act under which such regulatory review period occurred.

(g) 35 U.S.C. § 156(c)(4) - No other patent has been extended for the same regulatory review period for the product VPRIVTM.

(h) This application is being submitted within 60 days of regulatory agency approval.

(i) This application otherwise complies with all requirements of 35 U.S.C. § 156 and all applicable rules and procedures.

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Inventors: Peter Francis Daniel

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Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
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(12)(ii) Applicant respectfully submits that the length of the extension of patent term for U.S. Patent No. 7,138,262 B1 is 687 days pursuant to 35 U.S.C. § 156(c).

The length of the extension was determined pursuant to 37 C.F.R. § 1.775 as follows (the remainder of this section (12)(ii) is numbered so as to correspond to the numbering in 37 C.F.R. § 1.775):

(c) The regulatory review period under 35 U.S.C. § 156(g)(1)(B) is a total of 2,110 days, which is the sum of (1) and (2) below:

(1) The period of review under 35 U.S.C. § 156(g)(1)(B)(i), which is the number of days in the period beginning on the date the exemption became effective (May 20, 2004) and ending on the date an application was initially submitted (August 31, 2009), which is 1,930 days; and

(2) The period of review under 35 U.S.C. § 156(g)(1)(B)(ii), which is the number of days in the period beginning on the date the application was initially submitted (August 31, 2009) and ending on the date such application was approved (February 26, 2010), which is 180 days.

(d) The term of the patent as extended for a human drug, antibiotic drug or human biological product is determined by:

(1) Subtracting from the number of days determined to be in the regulatory review period, which is 2,110:

(i) The number of days in the regulatory review period which were on or before the date on which the patent issued (November 21, 2006) which is 916 days; and

(ii) The number of days in the period of (c)(1) and (c)(2) above during which applicant did not act with due diligence, which is zero (0) days; and

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(iii) One-half the number of days determined in subparagraph (c)(1) above after that period is reduced by subparagraph (d)(1)(i) and (d)(1)(ii) which, is $(1,930-916-0)/2$, or 507 days.

Thus, the number of days determined in subparagraph (c) above (2,110) is reduced by 1,423 $(916+507)$ days, for a total of 687 days;

(2) Adding the number of days as determined in subparagraph (d)(1), (687 days), to the original term of the patent (August 18, 2020) which results in the date of July 6, 2022.

(3) By adding fourteen (14) years to the date of approval of the New Drug Application (NDA 22575) which results in the date of February 26, 2024;

(4) By comparing the dates for the ends of the periods obtained pursuant to paragraphs (d)(2) and (d)(3) and selecting the earlier, which is July 6, 2022;

(5) (i) Since U.S. Patent No. 7,138,262 B1 issued after September 24, 1984, by adding 5 years to the original expiration date of the patent or any earlier date set by terminal disclaimer, which is August 18, 2025; and (ii) By comparing the dates obtained pursuant to paragraphs (d)(4) and (d)(5)(i) of this section with each other and selecting the earlier date, which is July 6, 2022.

Thus, the patent is entitled to extension until July 6, 2022.

(13) Statement Pursuant to 37 C.F.R. § 1.740(a)(13)

Applicant acknowledges a duty to disclose to the Director of the United States Patent and Trademark Office and the Secretary of Health and Human Services any information which is material to the determination of entitlement to the extension sought, e.g., as that duty is defined in 37 C.F.R. § 1.765.

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Inventors: Peter Francis Daniel

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(14) Applicable Fee [1.740(a)(14)]

The prescribed fee for receiving and acting upon this application is attached as a check in the amount of \$1,120.00. The Director is authorized to charge any additional fees required by this application to Deposit Account No. 50/2762, referencing attorney docket number S2071-701019.


(15) Name and Address for Correspondence [1.740(a)(15)]

All correspondence and inquiries may be directed to the undersigned, whose address, telephone number and fax number are as follows:

Laurie Butler Lawrence
Lando & Anastasi, LLP
One Main Street
Cambridge, MA 02142
Phone: 617-395-7000
Fax: 617-395-7070

Enclosed is a certification that the application for extension of patent term under 35 U.S.C. § 156 including its attachments and supporting papers is being submitted as one original and two (2) copies thereof (Attachment K) in compliance with 37 C.F.R. § 1.740(b).

Respectfully submitted,

By: 
Laurie Butler Lawrence, Reg. No. 46,593
LANDO & ANASTASI, LLP
One Main Street
Cambridge, Massachusetts 02142
United States of America
Telephone: 617-395-7000
Facsimile: 617-395-7070

In re U.S. Patent No.: 7,138,262 B1
Issued: November 21, 2006
Inventors: Peter Francis Daniel
Assignee: Shire Human Genetic Therapies,
Inc.
Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attorney Docket No.: S2071-701019/0013US

Date: April 22, 2010

Attachments:

- Power of Attorney (Attachment A)
- Package Insert for VPRIVTM (Attachment B)
- NDA approval letter from FDA to Shire Human Genetic Therapies, Inc., dated February 26, 2010 (with enclosure) (Attachment C)
- U.S. Patent No. 7,138,262 B1 (Attachment D)
- Maintenance Fee Statement (Attachment E)
- Brumshtein et al. (2010) Glycobiology 20(1):24-32 (Attachment F)
- Letter from FDA to Transkaryotic Therapies, Inc., dated January 12, 2004, providing the IND number and showing the date of receipt by FDA of the IND (Attachment G)
- A written record of the discussion that occurred on January 28, 2004 regarding modification of the protocol (Attachment G1)
- Letter from Transkaryotic Therapies, Inc. to FDA dated March 11, 2004, concerning amendment of protocol (Attachment G2)
- FDA communication to Transkaryotic Therapies, Inc., dated May 20, 2004, concerning amendment to protocol (Appendix G3).
- Letter from FDA to Shire Human Genetics Therapies, Inc. indicating the date the IND was put on clinical hold (Attachment H)
- Letter from FDA to Shire Human Genetics Therapies, Inc., dated December 21, 2006, removing the clinical hold and indicating that the protocol can be initiated (Attachment I)
- Letter from FDA to Shire Human Genetics Therapies, Inc., dated September 14, 2009, acknowledging receipt of the final submission of the NDA (Attachment J)
- Certification of Copies of Application Papers (Attachment K)

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

**Assignee: Shire Human Genetic Therapies,
Inc.**

**Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS**

Attachment A

Power of Attorney

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REVOCATION OF PRIOR POWERS OF ATTORNEY
and
NEW POWER OF ATTORNEY

Sir:

The undersigned, Shire Human Genetic Therapies, Inc., a Delaware Corporation, assignee of the entire right, title and interest for all of the patents and patent applications identified in the attached Schedule A, hereby revokes all previous powers of attorney or authorizations of agent given in the identified patents and patent applications and in any divisional, continuing, substitute, renewal, reexamination, or reissue applications thereof, and appoints all practitioners of Lowrie, Lando & Anastasi, LLP associated with Customer Number:

37462

as assignee's attorneys or agents with full power of substitution to take any and all action necessary with regard to the identified patents and patent applications, and with regard to any divisional, continuing, substitute, renewal or reissue applications thereof.

Please address all telephone calls to Laurie Butler Lawrence at telephone no. (617) 395-7000.

Please forward all correspondence to the correspondence address associated with

Customer Number:

37462

Shire Human Genetic Therapies, Inc.

By:

Name: Kerry A. Flynn

Title: Vice President, Intellectual Property

Dated:

April 14, 2008

ASSIGNEE CERTIFICATION

Attached to this power is a Certificate Under 37 CFR 3.73(b).

Dated:

April 17, 2008

Natalie A. Lissy
Natalie A. Lissy, Reg. No. 59,651
LOWRIE, LANDO & ANASTASI, LLP
Riverfront Office Park
One Main Street
Cambridge, MA 02142
(617) 395-7000

SCHEDULE A

U.S. Patents:

<u>U.S. PATENT NO.</u>	<u>ISSUE DATE</u>	<u>ATTORNEY'S DOCKET NO.</u>
6,924,365	08/02/2005	S2071-700410
7,229,793	06/12/2007	S2071-700719
6,569,681	05/27/2003	S2071-700919
7,138,262	11/21/2006	S2071-701019
5,965,125	10/12/1999	S2071-701419
6,472,181	10/29/2002	S2071-701440
6,582,391	06/24/2003	S2071-701441
6,083,725	07/04/2000	S2071-701510
6,566,099	05/20/2003	S2071-701520
7,122,354	10/17/2006	S2071-701521
6,395,884	05/28/2002	S2071-701540
5,817,789	10/06/1998	S2071-701619
6,027,921	02/22/2000	S2071-701640
6,262,026	07/17/2001	S2071-701641
6,858,578	02/22/2005	S2071-701642
6,419,920	07/16/2002	S2071-701730
6,458,574	10/01/2002	S2071-702030

SCHEDULE A

U.S. Patent Applications:

<u>U.S. APPLICATION NO.</u>	<u>FILING DATE</u>	<u>ATTORNEY'S DOCKET NO.</u>
11/581,979	10/17/2006	S2071-701040
11/028,850	01/03/2005	S2071-701620
10/160,452	05/31/2002	S2071-701740
10/165,060	07/07/2002	S2071-702040
11/403,618	04/13/2006	S2071-702540
11/671,588	02/06/2007	S2071-702719
10/775,678	02/10/2004	S2071-702810
08/712,614	09/13/1996	S2071-703119
10/423,225	04/25/2003	S2071-702510
09/686,497	10/11/2000	S2071-701319
11/924,804	10/26/2007	S2071-701320
11/925,125	10/26/2007	S2071-701321
11/925,167	10/26/2007	S2071-701322
11/928,247	10/30/2007	S2071-701323
10/165,968	06/10/2002	S2071-702020
60/375,584	04/25/2002	S2071-702500
60/771,555	02/07/2006	S2071-702700
10/968,870	10/18/2004	S2071-701020

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

STATEMENT UNDER 37 CFR 3.73(b)Applicant/Patent Owner: Peter Francis Daniel et al.Application No./Patent No.: 7,138,262 Filed/Issue Date: 11/21/2006Entitled: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH MANNOSE PROTEINSShire Human Genetics Therapies, Inc., a Delaware Corporation
(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that it is:

1. ☒ the assignee of the entire right, title, and interest; or
2. ☐ an assignee of less than the entire right, title and interest
(The extent (by percentage) of its ownership interest is _____ %)

in the patent application/patent identified above by virtue of either:

- A. ☐ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel _____, Frame _____, or for which a copy thereof is attached.

OR

- B. ☒ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: Peter Francis Daniel et al. To: Transkaryotic Therapies, Inc.
The document was recorded in the United States Patent and Trademark Office at
Reel 011662, Frame 0815, or for which a copy thereof is attached.
2. From: Transkaryotic Therapies, Inc. To: Shire Human Genetics Therapies, Inc.
The document was recorded in the United States Patent and Trademark Office at
Reel 018224, Frame 0390, or for which a copy thereof is attached.
3. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at
Reel _____, Frame _____, or for which a copy thereof is attached.

☐ Additional documents in the chain of title are listed on a supplemental sheet.

- ☒ As required by 37 CFR 3.73(b)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (i.e., a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

<u>/Natalie A. Lissy/</u>	<u>April 24, 2008</u>
Signature	Date
<u>Natalie A. Lissy, Reg. No. 59,651</u>	<u>617-395-7000</u>
Printed or Typed Name	Telephone Number
<u>Attorney</u>	
Title	

This collection of information is required by 37 CFR 3.73(b). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

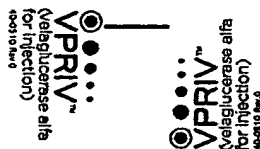
Inventors: Peter Francis Daniel

**Assignee: Shire Human Genetic Therapies,
Inc.**

**Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS**

Attachment B

Package Insert for VPRIV™



HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use VPRIV safely and effectively. See full prescribing information for VPRIV.

VPRIV (velaglucosamine alfa for injection)
Initial U.S. Approval: 2010

INDICATIONS AND USAGE

VPRIV (velaglucosamine alfa for injection) is a hydrolytic lysosomal glucocerebrosidase-specific enzyme indicated for long-term enzyme replacement therapy (ERT) for patients and adult patients with type 1 Gaucher disease (1).

DOSE AND ADMINISTRATION

- 60 Units/kg administered every other week as a 60-minute intravenous infusion (2).
- Patients currently being treated with lipoglucosamine for Gaucher disease can be switched to VPRIV. Patients previously treated on a stable dose of lipoglucosamine are recommended to begin treatment with VPRIV at that same dose when they switch from lipoglucosamine to VPRIV (2).
- Physicians can make dosage adjustments based on achievement and maintenance of each patient's therapeutic goals. Clinical trials have evaluated doses ranging from 15 Units/kg to 60 Units/kg every other week (2).

DOSE FORMS AND STRENGTHS

- Lyophilized powder to be reconstituted and diluted for infusion (3).
- Available in 200 Units and 400 Units single-use vials (3).

CONTRAINDICATIONS

- None (4).

WARNINGS AND PRECAUTIONS

- Hypersensitivity reactions: Treatment with VPRIV should be carefully re-evaluated in the presence of significant evidence of hypersensitivity to the product (5, 1).
- Infection-related reactions (5, 2).

ADVERSE REACTIONS

- The most common adverse reactions during clinical studies were infection-related reactions (5, 2, 6, 1).
- Other commonly observed adverse reactions in a 10% of patients were: headache, dizziness, abdominal pain, nausea, back pain, joint pain, upper respiratory tract infection, ecchymosis, PPT, pharyngitis, fatigue, and pyrexia (6, 1).
- To report SUSPECTED ADVERSE REACTIONS, contact Shire Human Genetic Therapies, Inc. at the DrugWatch™ phone 800-555-0680 or MedWatch@shire.com, or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch. See Section 17 for PATIENT COUNSELING INFORMATION.

Revised: 02/2010

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3. DOSAGE FORMS AND STRENGTHS
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*Sections or subsections omitted from the full prescribing information are not listed.

VPRIV™ (velaglucosamine alfa for injection)

FULL PRESCRIBING INFORMATION

1. INDICATIONS AND USAGE

VPRIV (velaglucosamine alfa for injection) is a hydrolytic lysosomal glucocerebrosidase-specific enzyme indicated for long-term enzyme replacement therapy (ERT) for patients and adult patients with type 1 Gaucher disease.

2. DOSAGE AND ADMINISTRATION

2.1 Recommended Dose

The recommended dose is 60 Units/kg administered every other week as a 60-minute intravenous infusion. Patients currently being treated with lipoglucosamine for type 1 Gaucher disease may be switched to VPRIV. Patients previously treated on a stable dose of lipoglucosamine are recommended to begin treatment with VPRIV at that same dose when they switch from lipoglucosamine to VPRIV.

Dose adjustments can be made based on achievement and maintenance of each patient's therapeutic goals. Clinical trials have evaluated doses ranging from 15 Units/kg to 60 Units/kg every other week. VPRIV should be administered under the supervision of a healthcare professional.

2.2 Preparation and Administration Instructions

The aseptic technique

VPRIV is a lyophilized powder, which requires reconstitution and dilution, and is intended for intravenous infusion only. VPRIV contains no preservatives and vials are single-use only. Discard any unused solution. VPRIV should be prepared as follows:

Determine the number of vials to be reconstituted based on the individual patient's weight and the prescribed dose. Follow the instructions in Table 1 for reconstitution.

Table 1: Reconstitution Instructions

	200 Units/vial	400 Units/vial
Volumes of Sterile Water for Injection, USP for reconstitution	2.2 mL	4.3 mL
Concentration after reconstitution	100 Units/mL	100 Units/mL
Withdrawal volume	2 mL	4 mL

Upon reconstitution, add vials gently. DO NOT SHAKE. Prior to further dilution, visually inspect the solution in the vials; the solution should be clear to slightly opalescent and colorless. Do not use if the solution is discolored or if flocculi/particulate matter is present. Withdraw the calculated volume of drug from the appropriate number of vials and dilute the total volume required in 600 mL of 0.9% sodium chloride solution suitable for IV administration. Mix gently. DO NOT SHAKE.

VPRIV should be administered over 60 minutes. VPRIV should not be mixed with other products in the same infusion tubing as the compatibility in solution with other products has not been evaluated. The diluted solution should be filtered through an in-line low protein-binding 0.2 µm filter during administration.

As VPRIV contains no preservatives, use reconstituted the product should be used immediately if immediate use is not possible, the reconstituted or diluted product may be stored for up to 24 hours at 2 to 8°C (36 to 46°F). Do not freeze. Protect from light. The infusion should be completed within 24 hours of reconstitution of vials.

3. DOSAGE FORMS AND STRENGTHS

VPRIV is a sterile, white to off-white, lyophilized powder for reconstitution with Sterile Water for Injection, USP, to yield a final concentration of 100 Units/mL.

VPRIV is available as 200 Units and 400 Units single-use vials.

4. CONTRAINDICATIONS

None.

5. WARNINGS AND PRECAUTIONS

5.1 Hypersensitivity Reactions

Hypersensitivity reactions have been reported in patients in clinical studies with VPRIV (see Adverse Reactions (6, 1)). As with any lysosomal enzyme product, hypersensitivity reactions are possible, therefore appropriate medical support should be readily available when VPRIV is administered. If a severe reaction occurs, suspend medical treatment for emergency treatment and to be followed.

Treatment with VPRIV should be approached with caution in patients who have exhibited symptoms of hypersensitivity to the active ingredient or excipients in the drug product or to other enzyme replacement therapy.

5.2 Infection-related Reactions

Infection-related reactions were the most commonly observed adverse reactions in patients treated with VPRIV in clinical studies. The most commonly observed symptoms of infection-related reactions were: headache, dizziness, hypertension, pyrexia, nausea, fatigue, and pharyngitis. Generally, the infection-related reactions were mild and self-limiting, and most patients recovered within the first 6 months of treatment and tended to occur less frequently with time.

The management of infection-related reactions should be based on the severity of the reaction, e.g., slowing the infusion rate, treatment with medications such as anticholinergics, antipyretics and/or corticosteroids, and/or stopping and resuming treatment with lowered infusion rate.

Pre-treatment with anticholinergic and/or corticosteroids may prevent subsequent reactions in those cases where symptomatic treatment was required. Patients were not routinely pre-medicated prior to infusion of VPRIV during clinical studies.

6. ADVERSE REACTIONS

6.1 Clinical Studies Experience

The data described below reflect exposure of 94 patients with type 1 Gaucher disease who received VPRIV at doses ranging from 15 Units/kg to 60 Units/kg every other week in 3 clinical studies. Patients were not given ERT and received VPRIV for 6 months and 40 patients received lipoglucosamine in VPRIV treatment and received VPRIV for 12 months (see Clinical Studies (14)). Patients were between 4 and 71 years old at time of first treatment with VPRIV, and included 46 male and 48 female patients.

The most common adverse reactions in patients treated with VPRIV were hypersensitivity reactions (see Warnings and Precautions (5, 1)).

The most commonly reported adverse reactions (occurring in ≥10% of patients) that were considered related to VPRIV are shown in Table 2. The most common adverse reactions were infection-related reactions.

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trial of a drug cannot be directly compared to rates in the clinical trial of another drug and may not reflect the rates observed in practice.

Table 2: Adverse Reactions Observed in ≥10% of Patients with Type 1 Gaucher Disease Treated with VPRIV

System Organ Class Preferred Term	Waste to ERT N = 54	Switched from lipoglucosamine to VPRIV N = 40
	Number of Patients (%)	
Respiratory system disorders		
Nasopharyngitis	19 (35.2)	12 (30)
Dysphagia	12 (22.2)	3 (7.5)
Gastrointestinal disorders		
Abdominal pain	10 (18.5)	6 (15)
Nausea	3 (5.6)	4 (10)
Musculoskeletal and connective tissue disorders		
Back pain	9 (16.7)	7 (17.5)
Joint pain (arthralgia)	5 (9.3)	3 (7.5)
Infections and infestations		
Upper respiratory tract infection	17 (31.5)	12 (30)
Investigations		
Activated partial thromboplastin time prolonged	6 (11.1)	2 (5)
General disorders and administration site conditions		
Infection-related reaction*	29 (53.7)	9 (22.5)
Pyrexia	12 (22.2)	5 (12.5)
Asthenia/fatigue	7 (13)	5 (12.5)

*Determine any event considered related to and occurring within up to 24 hours of VPRIV infusion.

Less common adverse reactions affecting more than one patient (≥1% in the treatment-emergent group and ≥2% in patients switched from lipoglucosamine to VPRIV treatment) were: back pain, dysphagia, rash, urticaria, flushing, hypertension, and pyrexia.

Pediatric Patients

All adverse reactions to VPRIV are considered related to pediatric patients (ages 4 to 17 years). Adverse reactions more commonly seen in pediatric patients compared to adult patients include (≥10% difference) upper respiratory tract infection, rash, PPT, pharyngitis, and pyrexia.

Immunogenicity

As with all therapeutic proteins, there is a potential for immunogenicity. In clinical studies, 1 of 54 treatment-naïve patients treated with VPRIV developed IgG class antibodies to VPRIV. In this patient, the antibodies were determined to be neutralizing in an in vitro assay. The infection-related reactions were reported for this patient. It is unknown if the presence of IgG antibodies to VPRIV is associated with a higher risk of infection reactions. Patients with an immune response to other enzyme replacement therapies who are switching to VPRIV should continue to be monitored for antibodies.

Immunogenicity assay results were highly dependent on the sensitivity and specificity of the assay. Additionally, the observed incidence of antibody positivity in an assay may be influenced by several factors, including assay methodology, sample handling, timing of sample collection, concomitant medications, and underlying disease. For these reasons, comparison of the incidence of antibodies to VPRIV with the incidence of antibodies to other products may be misleading.

7. DRUG INTERACTIONS

No drug-drug interaction studies have been conducted.

8. USE IN SPECIFIC POPULATIONS

8.1 Pregnancy - Category B

Reproductive studies with velaglucosamine alfa have been performed in pregnant rats at intravenous doses up to 17 mg/kg/day (102 mg/kg/day, about 1.8 times the recommended human dose of 60 Units/kg/day or 1.5 mg/kg/day or 55.5 mg/kg/day based on the body surface area). Reproductive studies have been performed in pregnant rabbits at intravenous doses up to 20 mg/kg/day (240 mg/kg/day, about 4.3 times the recommended human dose of 60 Units/kg/day based on the body surface area). These studies did not reveal any evidence of teratogenicity or harm to the fetus due to velaglucosamine alfa.

A pre- and post-natal development study in rats showed no evidence of any adverse effect on pre- and post-natal development at doses up to 17 mg/kg (102 mg/kg/day, about 1.8 times the recommended human dose of 60 Units/kg/day based on the body surface area). There are, however, no adequate and well-controlled studies in pregnant women. Because animal reproduction studies are not always predictive of human response, VPRIV should be used during pregnancy only if clearly needed.

8.2 Nursing Mothers

There are no data from studies in lactating women. It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when VPRIV is administered to a nursing woman.

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

**Assignee: Shire Human Genetic Therapies,
Inc.**

**Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS**

Attachment C

**NDA approval letter from FDA to Shire Human Genetic Therapies, Inc., dated
February 26, 2010 (with enclosure)**



DEPARTMENT OF HEALTH AND HUMAN SERVICES

Food and Drug Administration
Silver Spring MD 20993

NDA 022575

NDA APPROVAL

Shire Human Genetic Therapies, Inc.
Attention: Nikhil Mehta, Ph.D.
Vice President, Global Regulatory Affairs
700 Main Street
Cambridge, MA 02139

Dear Dr. Mehta:

Please refer to your new drug application (NDA) dated August 31, 2009, received August 31, 2009, submitted under section 505(b) of the Federal Food, Drug, and Cosmetic Act for VPRIV (velaglucerase alfa for injection).

We acknowledge receipt of your submissions dated July 30, August 31, September 17, 22, and 28, October 1, 9, 12, 23, and 29, November 16 and 20, December 1, 4, 15, 18, 22, and 31, 2009, and January 13, 14, 15, 26, and 27, and February 1, 8, 9, 17, 19, and 25, 2010.

This new drug application provides for the use of VPRIV (velaglucerase alfa for injection) for long-term enzyme replacement therapy (ERT) for pediatric and adult patients with type 1 Gaucher disease.

We have completed our review of this application, as amended. It is approved, effective on the date of this letter, for use as recommended in the enclosed agreed-upon labeling text.

Your application was not referred to an advisory committee because this drug is not the first in its class, the clinical study design was acceptable, the application did not raise significant safety or efficacy issues, the application did not raise significant public health questions on the role of the drug in the diagnosis, cure, mitigation, treatment or prevention of a disease, and outside expertise was not necessary.

CONTENT OF LABELING

As soon as possible, but no later than 14 days from the date of this letter, please submit the content of labeling [21 CFR 314.50(l)] in structured product labeling (SPL) format, as described at <http://www.fda.gov/ForIndustry/DataStandards/StructuredProductLabeling/default.htm>, that is identical to the submitted labeling (package insert submitted February 25, 2010). For administrative purposes, please designate this submission, "SPL for approved NDA 022575."

CARTON AND IMMEDIATE CONTAINER LABELS

We acknowledge your February 19, 2010, submission containing final printed carton and container labels.

Marketing the product with final printed labeling that is not identical to the approved labeling text may render the product misbranded and an unapproved new drug.

REQUIRED PEDIATRIC ASSESSMENTS

Under the Pediatric Research Equity Act (PREA) (21 U.S.C. 355c), all applications for new active ingredients, new indications, new dosage forms, new dosing regimens, or new routes of administration are required to contain an assessment of the safety and effectiveness of the product for the claimed indication in pediatric patients unless this requirement is waived, deferred, or inapplicable.

Because this drug product for this indication has an orphan drug designation, you are exempt from this requirement.

POSTMARKETING COMMITMENTS SUBJECT TO REPORTING REQUIREMENTS UNDER SECTION 506B

We remind you of your postmarketing commitments in your submission dated February 25, 2010. These commitments are listed below.

- 1600-01 Shire commits to utilize an antibody screening cut point based on a mean + 1.645 standard deviation for assay values from treatment naïve Gaucher patients. Shire will utilize the same methodology to calculate the anti-imiglucerase ECL cut point.

Final Report Submission: May 31, 2010

- 1600-02 Shire commits to revise the cut point for the confirmatory anti-velaglucerase and anti-imiglucerase screening assays to a level that is less than or equal to the cut point of the screening assay.

Final Report Submission: May 31, 2010

- 1600-03 Shire commits to re-assess the IgE cut point for the current ECL methodology using a chemically synthesized hybrid control. Shire commits to support assay validation using patient baseline values.

Final Report Submission: May 31, 2010

- 1600-04 Shire commits to develop an assay to measure the ability of patient antibodies to block the uptake of velaglucerase and imiglucerase into target cells.

Final Report Submission: November 30, 2010

- 1600-05 Shire commits to re-analyze all archived pharmacokinetic (PK) samples for Study TKT032 (using adequate in-process quality controls and standard curves) and recalculate velaglucerase alfa PK parameters.

Study Completion Date: May 31, 2010
Final Report Submission: June 30, 2010

- 1600-06 Shire commits to conduct a prospective PK study in patients with Type 1 Gaucher disease in the case that Shire fails to adequately characterize velaglucerase alfa PK using the archived PK samples (discussed under PMC #1600-05 above).

Final Protocol Submission: December 31, 2010
Study Completion Date: March 31, 2013
Final Report Submission: September 30, 2013

POSTMARKETING COMMITMENTS NOT SUBJECT TO THE REPORTING REQUIREMENTS UNDER SECTION 506B

We remind you of your postmarketing commitments in your submission dated February 25, 2010. These commitments are listed below.

- 1600-07 Shire commits to develop and implement a kinetic assay with a physiologically relevant substrate for drug substance and drug product release and stability testing. Results and specifications will be included in the final report.

Final Report Submission: December 31, 2011

- 1600-08 Shire commits to develop and implement a quantitative method that measures total carbohydrate content. Results and specifications will be included in the final report.

Final Report Submission: February 28, 2011

- 1600-09 Shire commits to replace the non-quantitative SDS-PAGE Silver stain method with a quantitative SDS-PAGE Coomassie test. Results and specifications will be included in the final report.

Final Report Submission: February 28, 2011

- 1600-10 Shire commits to demonstrate that Long R3 IGF1 is well controlled to ensure no impact on product quality. The results will be included in the final report.

Final Report Submission: February 28, 2011

- 1600-11 Shire commits to demonstrate the clearance capability of the process to remove hydrocortisone through hydrocortisone spike studies. The results will be included in the final report.

Final Report Submission: November 30, 2010

- 1600-12 Shire commits to re-evaluate drug substance and drug product release and stability specifications. Shire will submit the revised specifications and supporting data in the final report.

Final Report Submission: December 31, 2011

- 1600-13 Shire commits to update the specifications for SEC, RP-HPLC, and the glycan map, and to include acceptance criteria for the leading shoulder in SEC-HPLC, for peaks A and B in RP-HPLC, and for peak group 2 in the glycan map.

Final Report Submission: July 1, 2010

- 1600-14 Shire commits to update the peptide map specification using new acceptance criteria to reflect control of impurities. Shire commits to add the peptide map as a drug substance and drug product release and stability test with the new acceptance criteria.

Final Report Submission: July 1, 2010

- 1600-15 Shire commits to include the cellular uptake bioassay for drug product release testing.

Final Report Submission: April 1, 2010

- 1600-16 Shire commits to provide a report containing the sub-visible particulates (2 – 10 μ m) analyses, risk assessment and risk mitigation strategies.

Final Report Submission: September 30, 2010

- 1600-17 Shire commits to include drug substance and drug product stress conditions in the annual stability program. The revised stability protocols will be included.

Final Protocol Submission: April 1, 2010

- 1600-18 Shire commits to evaluate the impact of pH on the in-use stability of the drug product and to provide assurance that procedures are in place to control this risk to product quality.

Final Protocol Submission: December 31, 2010

Submit clinical protocols to your IND 061220 for this product. Submit nonclinical and chemistry, manufacturing, and controls protocols and all study final reports to this NDA. In addition, under 21 CFR 314.81(b)(2)(vii) and 314.81(b)(2)(viii) you should include a status summary of each commitment in your annual report to this NDA. The status summary should include expected summary completion and final report submission dates, any changes in plans since the last annual report, and, for clinical studies/trials, number of patients entered into each study/trial. All submissions, including supplements, relating to these postmarketing commitments should be prominently labeled "Postmarketing Commitment Protocol," "Postmarketing Commitment Final Report," or "Postmarketing Commitment Correspondence."

PROMOTIONAL MATERIALS

You may request advisory comments on proposed introductory advertising and promotional labeling. To do so, submit, in triplicate, a cover letter requesting advisory comments, the proposed materials in draft or mock-up form with annotated references, and the package insert to:

Food and Drug Administration
Center for Drug Evaluation and Research
Division of Drug Marketing, Advertising, and Communications
5901-B Ammendale Road
Beltsville, MD 20705-1266

As required under 21 CFR 314.81(b)(3)(i), you must submit final promotional materials, and the package insert, at the time of initial dissemination or publication, accompanied by a Form FDA 2253. For instruction on completing the Form FDA 2253, see page 2 of the Form. For more information about submission of promotional materials to the Division of Drug Marketing, Advertising, and Communications (DDMAC), see <http://www.fda.gov/AboutFDA/CentersOffices/CDER/ucm090142.htm>.

Please submit one market package of the drug product when it is available.

LETTERS TO HEALTH CARE PROFESSIONALS

If you issue a letter communicating important safety-related information about this drug product (i.e., a "Dear Health Care Professional" letter), we request that you submit an electronic copy of the letter to both this NDA and to the following address:

MedWatch
Food and Drug Administration
Suite 12B-05
5600 Fishers Lane
Rockville, MD 20857

REPORTING REQUIREMENTS

We remind you that you must comply with reporting requirements for an approved NDA (21 CFR 314.80 and 314.81).

MEDWATCH-TO-MANUFACTURER PROGRAM

The MedWatch-to-Manufacturer Program provides manufacturers with copies of serious adverse event reports that are received directly by the FDA. New molecular entities and important new biologics qualify for inclusion for three years after approval. Your firm is eligible to receive copies of reports for this product. To participate in the program, please see the enrollment instructions and program description details at <http://www.fda.gov/Safety/MedWatch/HowToReport/ucm166910.htm>.

If you have any questions, call Wes Ishihara, Regulatory Project Manager, at (301) 796-0069.

Sincerely,

{See appended electronic signature page}

Julie Beitz, M.D.
Director
Office of Drug Evaluation III
Center for Drug Evaluation and Research

Enclosure: Package Insert

HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use VPRIV safely and effectively. See full prescribing information for VPRIV.

VPRIV™ (velaglu­cerase alfa for injection)
Initial U.S. Approval: 2010

INDICATIONS AND USAGE

VPRIV (velaglu­cerase alfa for injection) is a hydrolytic lysosomal glucocerebrosi­de-specific enzyme indicated for long-term enzyme replacement therapy (ERT) for pediatric and adult patients with type 1 Gaucher disease (1).

DOSAGE AND ADMINISTRATION

- 60 Units/kg administered every other week as a 60-minute intravenous infusion (2).
- Patients currently being treated with imigluc­erase for Gaucher disease can be switched to VPRIV. Patients previously treated on a stable dose of imigluc­erase are recommended to begin treatment with VPRIV at that same dose when they switch from imigluc­erase to VPRIV (2).
- Physicians can make dosage adjustments based on achievement and maintenance of each patient's therapeutic goals. Clinical trials have evaluated doses ranging from 15 Units/kg to 60 Units/kg every other week (2).

DOSAGE FORMS AND STRENGTHS

- Lyophilized powder to be reconstituted and diluted for infusion (3).
- Available in 200 Units and 400 Units single-use vials (3).

CONTRAINDICATIONS

- None (4).

WARNINGS AND PRECAUTIONS

- Hypersensitivity reactions: Treatment with VPRIV should be carefully re-evaluated in the presence of significant evidence of hypersensitivity to the product (5.1).
- Infusion-related reactions (5.2).

ADVERSE REACTIONS

- The most common adverse reactions during clinical studies were infusion-related reactions (5.2, 6.1).
- Other commonly observed adverse reactions in ≥ 10% of patients were: headache, dizziness, abdominal pain, nausea, back pain, joint pain, upper respiratory tract infection, activated PTT prolonged, fatigue/asthenia, and pyrexia (6.1).

To report SUSPECTED ADVERSE REACTIONS, contact Shire Human Genetic Therapies, Inc. at the OnePathSM phone # 1-866-888-0660 or MedInfoGlobal@Shire.com, or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch

See Section 17 for PATIENT COUNSELING INFORMATION.

Revised: 02/2010

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*Sections or subsections omitted from the full prescribing information are not listed.

VPRIV™ (velaglucerase alfa for injection)

FULL PRESCRIBING INFORMATION

1 INDICATIONS AND USAGE

VPRIV (velaglucerase alfa for injection) is a hydrolytic lysosomal glucocerebroside-specific enzyme indicated for long-term enzyme replacement therapy (ERT) for pediatric and adult patients with type 1 Gaucher disease.

2 DOSAGE AND ADMINISTRATION

2.1 Recommended Dose

The recommended dose is 60 Units/kg administered every other week as a 60-minute intravenous infusion.

Patients currently being treated with imiglucerase for type 1 Gaucher disease may be switched to VPRIV. Patients previously treated on a stable dose of imiglucerase are recommended to begin treatment with VPRIV at that same dose when they switch from imiglucerase to VPRIV.

Dosage adjustments can be made based on achievement and maintenance of each patient's therapeutic goals. Clinical studies have evaluated doses ranging from 15 Units/kg to 60 Units/kg every other week.

VPRIV should be administered under the supervision of a healthcare professional.

2.2 Preparation and Administration Instructions

Use aseptic technique

VPRIV is a lyophilized powder, which requires reconstitution and dilution, and is intended for intravenous infusion only. VPRIV contains no preservatives and vials are single-use only. Discard any unused solution. VPRIV should be prepared as follows:

Determine the number of vials to be reconstituted based on the individual patient's weight and the prescribed dose. Follow the instructions in Table 1 for reconstitution.

Table 1: Reconstitution Instructions

	200 Units/vial	400 Units/vial
Volume of Sterile Water for Injection, USP, for reconstitution	2.2 mL	4.3 mL
Concentration after reconstitution	100 Units/mL	100 Units/mL
Withdrawal volume	2 mL	4 mL

Upon reconstitution, mix vials gently. **DO NOT SHAKE.** Prior to further dilution, visually inspect the solution in the vials; the solution should be clear to slightly opalescent and colorless; do not use if the solution is discolored or if foreign particulate matter is present. Withdraw the calculated volume of drug from the appropriate number of vials and dilute the total volume required in 100 mL of 0.9% sodium chloride solution suitable for IV administration. Mix gently. **DO NOT SHAKE.**

VPRIV should be administered over 60 minutes. VPRIV should not be infused with other products in the same infusion tubing as the compatibility in solution with other products has not been evaluated. The diluted solution should be filtered through an in-line low protein-binding 0.2µm filter during administration.

As VPRIV contains no preservatives, once reconstituted the product should be used immediately. If immediate use is not possible, the reconstituted or diluted product may be stored for up to 24 hours at 2 to 8°C (36 to 46°F). Do not freeze. Protect from light. The infusion should be completed within 24 hours of reconstitution of vials.

3 DOSAGE FORMS AND STRENGTHS

VPRIV is a sterile, white to off-white, lyophilized powder for reconstitution with Sterile Water for Injection, USP, to yield a final concentration of 100 Units/mL.

VPRIV is available as 200 Units and 400 Units single-use vials.

4 CONTRAINDICATIONS

None.

5 WARNINGS AND PRECAUTIONS

5.1 Hypersensitivity Reactions

Hypersensitivity reactions have been reported in patients in clinical studies with VPRIV [*see Adverse Reactions (6.1)*]. As with any intravenous protein product, hypersensitivity reactions are possible, therefore appropriate medical support should be readily available when VPRIV is administered. If a severe reaction occurs, current medical standards for emergency treatment are to be followed.

Treatment with VPRIV should be approached with caution in patients who have exhibited symptoms of hypersensitivity to the active ingredient or excipients in the drug product or to other enzyme replacement therapy.

5.2 Infusion-related Reactions

Infusion-related reactions were the most commonly observed adverse reactions in patients treated with VPRIV in clinical studies. The most commonly observed symptoms of infusion-related reactions were: headache, dizziness, hypotension, hypertension, nausea, fatigue/asthenia, and pyrexia. Generally the infusion-related reactions were mild and, in treatment-naïve patients, onset occurred mostly during the first 6 months of treatment and tended to occur less frequently

with time.

The management of infusion-related reactions should be based on the severity of the reaction, e.g. slowing the infusion rate, treatment with medications such as antihistamines, antipyretics and/or corticosteroids, and/or stopping and resuming treatment with increased infusion time.

Pre-treatment with antihistamines and/or corticosteroids may prevent subsequent reactions in those cases where symptomatic treatment was required. Patients were not routinely pre-medicated prior to infusion of VPRIV during clinical studies.

6 ADVERSE REACTIONS

6.1 Clinical Studies Experience

The data described below reflect exposure of 94 patients with type 1 Gaucher disease who received VPRIV at doses ranging from 15 Units/kg to 60 Units/kg every other week in 5 clinical studies. Fifty-four (54) patients were naïve to ERT and received VPRIV for 9 months and 40 patients switched from imiglucerase to VPRIV treatment and received VPRIV for 12 months [see *Clinical Studies (14)*]. Patients were between 4 and 71 years old at time of first treatment with VPRIV, and included 46 male and 48 female patients.

The most serious adverse reactions in patients treated with VPRIV were hypersensitivity reactions [see *Warnings and Precautions (5.1)*].

The most commonly reported adverse reactions (occurring in $\geq 10\%$ of patients) that were considered related to VPRIV are shown in Table 2. The most common adverse reactions were infusion-related reactions.

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice.

Table 2: Adverse Reactions Observed in $\geq 10\%$ of Patients with Type 1 Gaucher Disease Treated with VPRIV

System Organ Class Preferred Term	Naïve to ERT N = 54	Switched from imiglucerase to VPRIV N = 40
	Number of Patients (%)	
Nervous system disorders		
Headache	19 (35.2)	12 (30)
Dizziness	12 (22.2)	3 (7.5)
Gastrointestinal disorders		
Abdominal pain	10 (18.5)	6 (15)
Nausea	3 (5.6)	4 (10)
Musculoskeletal and connective tissue disorders		
Back pain	9 (16.7)	7 (17.5)
Joint pain (knee)	8 (14.8)	3 (7.5)
Infections and infestations		
Upper respiratory tract infection	17 (31.5)	12 (30)
Investigations		
Activated partial thromboplastin time prolonged	6 (11.1)	2 (5)
General disorders and administration site conditions		
Infusion-related reaction*	28 (51.9)	9 (22.5)
Pyrexia	12 (22.2)	5 (12.5)
Asthenia/Fatigue	7 (13)	5 (12.5)
*Denotes any event considered related to and occurring within up to 24 hours of VPRIV infusion		

Less common adverse reactions affecting more than one patient ($>3\%$ in the treatment-naïve group and $>2\%$ in patients switched from imiglucerase to VPRIV treatment) were bone pain, tachycardia, rash, urticaria, flushing, hypertension, and hypotension.

Pediatric Patients

All adult adverse reactions to VPRIV are considered relevant to pediatric patients (ages 4 to 17 years). Adverse reactions more commonly seen in pediatric patients compared to adult patients include ($>10\%$ difference): upper respiratory tract infection, rash, aPTT prolonged, and pyrexia.

Immunogenicity

As with all therapeutic proteins, there is a potential for immunogenicity. In clinical studies, 1 of 54 treatment-naïve patients treated with VPRIV developed IgG class antibodies to VPRIV.

In this patient, the antibodies were determined to be neutralizing in an in vitro assay. No infusion-related reactions were reported for this patient. It is unknown if the presence of IgG antibodies to VPRIV is associated with a higher risk of infusion reactions. Patients with an immune response to other enzyme replacement therapies who are switching to VPRIV should continue to be monitored for antibodies.

Immunogenicity assay results are highly dependent on the sensitivity and specificity of the assay. Additionally, the observed incidence of antibody positivity in an assay may be influenced by several factors, including assay methodology, sample handling, timing of sample collection, concomitant medications, and underlying disease. For these reasons, comparison of the incidence of antibodies to VPRIV with the incidence of antibodies to other products may be misleading.

7 DRUG INTERACTIONS

No drug-drug interaction studies have been conducted.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy – Category B

Reproduction studies with velaglucerase alfa have been performed in pregnant rats at intravenous doses up to 17 mg/kg/day (102 mg/m²/day, about 1.8 times the recommended human dose of 60 Units/kg/day or 1.5 mg/kg/day or 55.5 mg/m²/day based on the body surface area). Reproduction studies have been performed in pregnant rabbits at intravenous doses up to 20 mg/kg/day (240 mg/m²/day, about 4.3 times the recommended human dose of 60 Units/kg/day based on the body surface area). These studies did not reveal any evidence of impaired fertility or harm to the fetus due to velaglucerase alfa.

A pre- and postnatal development study in rats showed no evidence of any adverse effect on pre- and postnatal development at doses up to 17 mg/kg (102 mg/m²/day, about 1.8 times the recommended human dose of 60 Units/kg/day based on the body surface area). There are, however, no adequate and well-controlled studies in pregnant women. Because animal reproduction studies are not always predictive of human response, VPRIV should be used during pregnancy only if clearly needed.

8.3 Nursing Mothers

There are no data from studies in lactating women. It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when VPRIV is administered to a nursing woman.

8.4 Pediatric Use

The safety and effectiveness of VPRIV have been established in patients between 4 and 17 years of age. Use of VPRIV in this age group is supported by evidence from adequate and well-controlled studies of VPRIV in adults and pediatric [20 of 94 (21%)] patients. The safety and efficacy profiles were similar between pediatric and adult patients [*see Adverse Reactions (6.1) and Clinical Studies (14)*]. The safety of VPRIV has not been established in pediatric patients

younger than 4 years of age.

8.5 Geriatric Use

During clinical studies 4 patients aged 65 or older were treated with VPRIV. Clinical studies of VPRIV did not include sufficient numbers of subjects aged 65 and over to determine whether they respond differently from younger subjects. Other reported clinical experience has not identified differences in responses between the elderly and younger patients. In general, dose selection for an elderly patient should be approached cautiously, considering potential comorbid conditions.

10 OVERDOSAGE

There is no experience with overdose of VPRIV.

11 DESCRIPTION

The active ingredient of VPRIV is velaglucerase alfa, which is produced by gene activation technology in a human fibroblast cell line. Velaglucerase alfa is a glycoprotein of 497 amino acids; with a molecular weight of approximately 63 kDa. Velaglucerase alfa has the same amino acid sequence as the naturally occurring human enzyme, glucocerebrosidase. Velaglucerase alfa contains 5 potential N-linked glycosylation sites; four of these sites are occupied by glycan chains. Velaglucerase alfa is manufactured to contain predominantly high mannose-type N-linked glycan chains. The high mannose type N-linked glycan chains are specifically recognized and internalized via the mannose receptor present on the surface on macrophages, the cells that accumulate glucocerebroside in Gaucher disease. Velaglucerase alfa catalyzes the hydrolysis of the glycolipid glucocerebroside to glucose and ceramide in the lysosome.

VPRIV is dosed by Units/kg, where one Unit of enzyme activity is defined as the quantity of enzyme required to convert one micromole of p-nitrophenyl β -D-glucopyranoside to p-nitrophenol per minute at 37°C.

VPRIV is supplied as a sterile, preservative free, lyophilized powder in single-use vials. Following reconstitution with Sterile Water for Injection, USP, the solution contains the components listed in Table 3.

Table 3: VPRIV Composition Following Reconstitution

	Extractable 200 Units/vial	Extractable 400 Units/vial
Active Ingredient		
velaglucerase alfa	200 Units	400 Units
Inactive Ingredients		
citric acid, monohydrate	2.52 mg	5.04 mg
polysorbate 20	0.22 mg	0.44 mg
sodium citrate, dihydrate	25.88 mg	51.76 mg
sucrose	100 mg	200 mg

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

Gaucher disease is an autosomal recessive disorder caused by mutations in the GBA gene, which results in a deficiency of the lysosomal enzyme beta-glucocerebrosidase. Glucocerebrosidase catalyzes the conversion of the sphingolipid glucocerebroside into glucose and ceramide. The enzymatic deficiency causes an accumulation of glucocerebroside primarily in the lysosomal compartment of macrophages, giving rise to foam cells or "Gaucher cells". In this lysosomal storage disorder (LSD), clinical features are reflective of the accumulation of Gaucher cells in the liver, spleen, bone marrow, and other organs. The accumulation of Gaucher cells in the liver and spleen leads to organomegaly. Presence of Gaucher cells in the bone marrow and spleen lead to clinically significant anemia and thrombocytopenia.

Velaglucerase alfa catalyzes the hydrolysis of glucocerebroside, reducing the amount of accumulated glucocerebroside.

12.3 Pharmacokinetics

In a multicenter study conducted in pediatric (N=7, 4 to 17 years old) and adult (N=15, 19 to 62 years old) patients with type 1 Gaucher disease, pharmacokinetic evaluations were performed at Weeks 1 and 37 following 60-minute intravenous infusions of VPRIV 60 Units/kg every other week. Serum velaglucerase alfa concentrations declined rapidly with a mean half life of 11 to 12 minutes. The mean velaglucerase alfa clearance ranged from 6.72 to 7.56 mL/min/kg. The mean volume of distribution at steady state ranged from 82 to 108 mL/kg (8.2% to 10.8% of body weight). However, because an inadequately validated analytical assay method was used in the evaluations, the accurate and definitive pharmacokinetic parameter values are not currently available.

No accumulation or change in velaglucerase alfa pharmacokinetics over time from Weeks 1 to 37 was observed upon multiple-dosing 60 Units/kg every other week.

Based on the limited data, there were no notable pharmacokinetic differences between male and female patients in this study. The effect of age on pharmacokinetics of velaglucerase alfa was inconclusive.

The effect of anti-drug antibody formation on the pharmacokinetic parameters of velaglucerase alfa is unknown.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

Long-term studies in animals to evaluate carcinogenic potential or studies to evaluate mutagenic potential have not been performed with velaglucerase alfa.

In a male and female fertility study in rats, velaglucerase alfa did not cause any significant adverse effect on male or female fertility parameters up to a maximum dose of 17 mg/kg/day (102 mg/m²/day, about 1.8 times the recommended human dose of 60 Units/kg/day based on the

body surface area).

14 CLINICAL STUDIES

The efficacy of VPRIV was assessed in three clinical studies in a total of 99 patients with type 1 Gaucher disease: 82 patients age 4 years and older received VPRIV and 17 patients age 3 years and older received imiglucerase. Studies I and II were conducted in patients who were not currently receiving Gaucher disease-specific therapy. Study III was conducted in patients who were receiving imiglucerase treatment immediately before starting VPRIV. In these studies, VPRIV was administered intravenously over 60 minutes at doses ranging from 15 Units/kg to 60 Units/kg every other week.

14.1 Studies of VPRIV as Initial Therapy

Study I was a 12-month, randomized, double-blind, parallel-dose-group, multinational study in 25 patients age 4 years and older with Gaucher disease-related anemia and either thrombocytopenia or organomegaly. Patients were not allowed to have had disease-specific therapy for at least the previous 30 months; all but one had no prior therapy. The mean age was 26 years and 60% were male. Patients were randomized to receive VPRIV at a dose of either 45 Units/kg (N=13) or 60 Units/kg (N=12) every other week.

At baseline, mean hemoglobin concentration was 10.6 g/dL, mean platelet count was $97 \times 10^9/L$, mean liver volume was 3.6 % of body weight (% BW), and mean spleen volume was 2.9 % BW. For all studies, liver and spleen volumes were measured by MRI. The changes in clinical parameters after 12 months of treatment are shown in Table 4. The observed change from baseline in the primary endpoint, hemoglobin concentration, was considered to be clinically meaningful in light of the natural history of untreated Gaucher disease.

Table 4: Mean Change from Baseline to Month 12 for Clinical Parameters in Patients with Type 1 Gaucher Disease Initiating Therapy with VPRIV in Study I

Clinical Parameter	Mean Changes from Baseline \pm Std. Err. of the Mean	
	VPRIV Dose (given every other week)	
	45 Units/kg N = 13	60 Units/kg N = 12
Hemoglobin concentration change (g/dL)	$2.4 \pm 0.4^*$	$2.4 \pm 0.3^{**}$
Platelet count change ($\times 10^9/L$)	$41 \pm 14^*$	$51 \pm 12^*$
Liver volume change (% BW)	-0.30 ± 0.29	-0.84 ± 0.33
Spleen volume change (% BW)	$-1.9 \pm 0.6^*$	$-1.9 \pm 0.5^*$

** Primary study endpoint was hemoglobin concentration change in the 60 Unit/kg group, $p < 0.001$

* Statistically significant changes from baseline after adjusting for performing multiple tests

Study II was a 9-month, randomized, double-blind, active-controlled (imiglucerase), parallel-group, multinational study in 34 patients age 3 years and older. Patients were required to have Gaucher disease-related anemia and either thrombocytopenia or organomegaly. Patients were

not allowed to have had disease-specific therapy for at least the previous 12 months. The mean age was 30 years and 53% were female; the youngest patient who received VPRIV was age 4 years. Patients were randomized to receive either 60 Units/kg of VPRIV (N=17) or 60 Units/kg of imiglucerase (N=17) every other week.

At baseline, the mean hemoglobin concentration was 11.0 g/dL, mean platelet count was $171 \times 10^9/L$, and mean liver volume was 4.3 % BW. For the patients who had not had splenectomy (7 in each group) the mean spleen volume was 3.4 % BW. After 9 months of treatment, the mean absolute increase from baseline in hemoglobin concentration was $1.6 \text{ g/dL} \pm 0.2 \text{ (SE)}$ for patients treated with VPRIV. The mean treatment difference in change from baseline to 9 months [VPRIV – imiglucerase] was $0.1 \text{ g/dL} \pm 0.4 \text{ (SE)}$.

In Studies I and II, examination of age and gender subgroups did not identify differences in response to VPRIV among these subgroups. The number of non-Caucasian patients in these studies was too small to adequately assess any difference in effects by race.

14.2 Study in Patients Switching from Imiglucerase Treatment to VPRIV

Study III was a 12-month, open-label, single-arm, multinational study in 40 patients age 9 years and older who had been receiving treatment with imiglucerase at doses ranging between 15 Units/kg to 60 Units/kg for a minimum of 30 consecutive months. Patients also were required to have a stable biweekly dose of imiglucerase for at least 6 months prior to enrollment. The mean age was 36 years and 55% were female. Imiglucerase therapy was stopped, and treatment with VPRIV was administered every other week at the same number of units as the patient's previous imiglucerase dose. Adjustment of dosage was allowed by study criteria if needed in order to maintain clinical parameters.

Hemoglobin concentrations and platelet counts remained stable on average through 12 months of VPRIV treatment. After 12 months of treatment with VPRIV the median hemoglobin concentration was 13.5 g/dL (range: 10.8, 16.1) vs. the baseline value of 13.8 g/dL (range: 10.4, 16.5), and the median platelet count after 12 months was $174 \times 10^9/L$ (range: 24, 408) vs. the baseline value of $162 \times 10^9/L$ (range: 29, 399). No patient required dosage adjustment during the 12-month treatment period.

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16 HOW SUPPLIED/STORAGE AND HANDLING

VPRIV is a sterile, preservative free, lyophilized powder requiring reconstitution and further dilution prior to use. It is supplied in individually packaged glass vials, which are closed with a butyl rubber stopper with a fluoro-resin coating and are sealed with an aluminum overseal with a flip-off plastic cap. The vials are intended for single use only. VPRIV is available as: 200 Units/vial NDC 54092-701-02 and 400 Units/vial NDC 54092-701-04.

16.1 Storage

VPRIV should be stored in a refrigerator at 2 to 8°C (36 to 46°F). Do not use VPRIV after the expiration date on the vial. Do not freeze.

Protect vial from light.

17 PATIENT COUNSELING INFORMATION

- VPRIV should be administered under the supervision of a healthcare professional. VPRIV is a treatment that is given intravenously (by IV) every other week. The infusion typically takes up to 60 minutes.
- Patients should be advised that VPRIV may cause hypersensitivity reactions or infusion-related reactions. Infusion-related reactions can usually be managed by slowing the infusion rate, treatment with medications such as antihistamines, antipyretics and/or corticosteroids, and/or stopping and resuming treatment with increased infusion time. Pre-treatment with antihistamines and/or corticosteroids may prevent subsequent reactions. Treatment with VPRIV should be carefully re-evaluated in the presence of significant evidence of hypersensitivity to the product [*see Warnings and Precautions (5.1, 5.2)*].

Rx Only

VPRIV is manufactured by:

Shire Human Genetic Therapies, Inc.
700 Main Street
Cambridge, MA 02139

OnePath is a service mark and VPRIV is a trademark of Shire Human Genetic Therapies, Inc.

Application
Type/Number

Submission
Type/Number

Submitter Name

Product Name

NDA-22575

ORIG-1

SHIRE HUMAN
GENETIC
THERAPIES INC

VELAGLUCERASE ALFA

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/s/

JULIE G BEITZ
02/26/2010

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment D

U.S. Patent No. 7,138,262 B1



US007138262B1

(12) United States Patent
Daniel**(10) Patent No.: US 7,138,262 B1****(45) Date of Patent: Nov. 21, 2006****(54) HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH MANNOSE PROTEINS****(75) Inventor: Peter Francis Daniel, Natick, MA (US)****(73) Assignee: Shire Human Genetic Therapies, Inc., Cambridge, MA (US)****(*) Notice:** Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.**(21) Appl. No.: 09/641,471****(22) Filed: Aug. 18, 2000****(51) Int. Cl.**

C12N 9/26 (2006.01)

C12P 19/28 (2006.01)

C12N 5/02 (2006.01)

C12N 5/06 (2006.01)

C12N 15/67 (2006.01)

C07H 21/04 (2006.01)

(52) U.S. Cl. 435/201; 435/85; 435/325; 435/358; 435/463; 536/23.2**(58) Field of Classification Search** 435/201, 435/325, 68.1, 69.1, 72, 85, 358, 365, 463; 536/23.2

See application file for complete search history.

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(57) ABSTRACT

The invention features a method of producing a high mannose glucocerebrosidase (hmGCB) which includes: providing a cell which is capable of expressing glucocerebrosidase (GCB), and allowing production of GCB having a precursor oligosaccharide under conditions which prevent the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB, to thereby produce an hmGCB preparation. Preferably, the condition which prevents the removal of at least one mannose residue distal to the pentasaccharide core is inhibition of a class 1 processing mannosidase and/or a class 2 processing mannosidase. The invention also features an hmGCB preparation and methods of using an hmGCB preparation.

63 Claims, 1 Drawing Sheet

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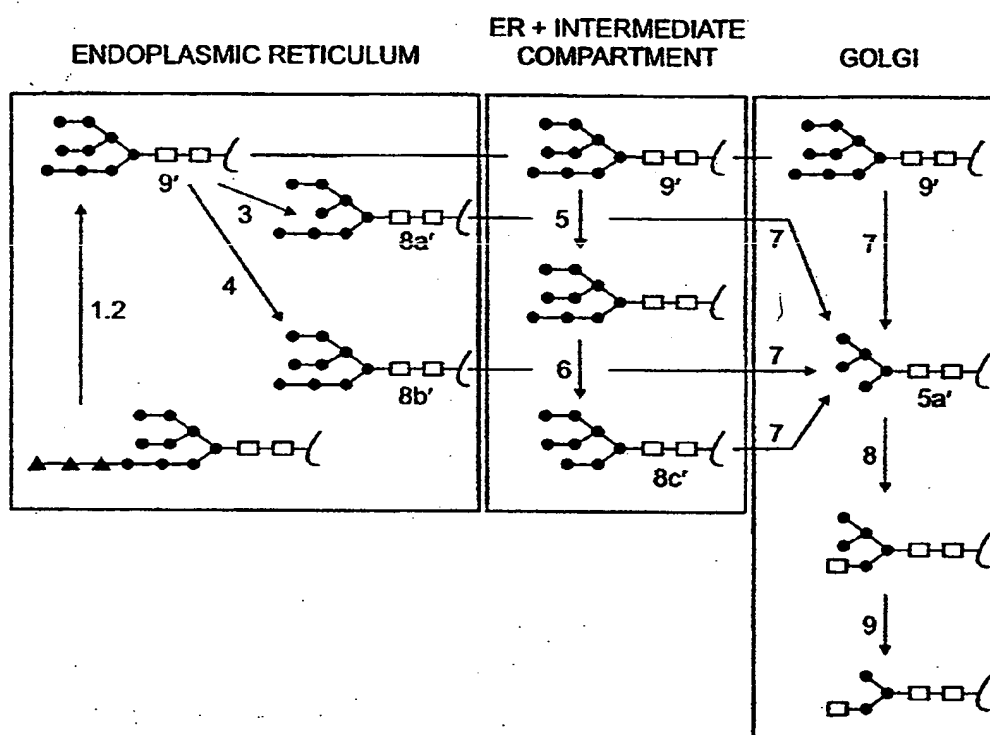


FIG. 1

1

HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH MANNOSE PROTEINS

BACKGROUND OF THE INVENTION

Gaucher disease is an autosomal recessive lysosomal storage disorder characterized by a deficiency in the lysosomal enzyme, glucocerebrosidase (GCB). GCB hydrolyzes the glycolipid glucocerebroside that is formed after degradation of glycosphingolipids in the membranes of white blood cells and red blood cells. The deficiency in this enzyme causes glucocerebroside to accumulate in large quantities in the lysosomes of phagocytic cells located in the liver, spleen and bone marrow of Gaucher patients. Accumulation of these molecules causes a range of clinical manifestations including splenomegaly, hepatomegaly, skeletal disorder, thrombocytopenia and anemia. (Beutler et al. Gaucher disease; In: The Metabolic and Molecular Bases of Inherited Disease (McGraw-Hill, Inc. New York, 1995) pp. 2625-2639)

Treatments for patients suffering from this disease include administration of analgesics for relief of bone pain, blood and platelet transfusions and, in some cases, splenectomy. Joint replacement is sometimes necessary for patients who experience bone erosion.

Enzyme replacement therapy with GCB has been used as a treatment for Gaucher disease. Current treatment of patients with Gaucher disease includes administration of a carbohydrate remodeled GCB derived from human placenta or Chinese hamster ovary (CHO) cells transfected with a GCB expression construct and known as α glucerase or imiglucerase, respectively. The treatment is extremely expensive in part because of the cost of removing sugars from GCB to expose the trimannosyl core of complex glycans in order to target the enzyme to mannose receptors on cells of reticuloendothelial origin. The scarcity of the human placental tissue (in the case of α glucerase), complex purification protocols, and the relatively large amounts of the carbohydrate remodeled GCB required all contribute to the cost of the treatment.

SUMMARY OF THE INVENTION

The invention is based, in part, on the discovery that by preventing removal of one or more mannose residues distal from the pentasaccharide core of a precursor oligosaccharide chain of a protein, e.g., a lysosomal storage enzyme, a high mannose protein such as high mannose glucocerebrosidase (hmGCB) can be obtained. These high mannose proteins can be used to target the protein to cells which express mannose receptors. Such cells can include cells of reticuloendothelial origin including macrophages, Kupffer cells and histiocytes. Thus, these high mannose proteins can be used, for example, to target delivery by receptor mediated endocytosis to lysosomes to treat various lysosomal storage diseases.

In particular, hmGCB has been found to efficiently target mannose receptors. Mannose receptors are present on macrophages and other cells, e.g., dendritic cells, cardiomyocytes and glial cells, and are instrumental in receptor-mediated endocytosis. The absence of GCB in patients with Gaucher disease leads to accumulation of glucocerebroside, primarily in cells of reticuloendothelial origin including macrophages, Kupffer cells and histiocytes. Because these cells express mannose receptors on their surface, hmGCB can be used to effectively target delivery of a corrective enzyme to the lysosomes through receptor-mediated

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endocytosis, thereby treating Gaucher disease. Surprisingly, it was found that hmGCB uptake by macrophages was increased as compared to uptake of GCB secreted from cells.

Accordingly, in one aspect, the invention features a method of producing a preparation of high mannose glucocerebrosidase (hmGCB). The method includes:

providing a cell which is capable of expressing GCB; and allowing production of GCB having a precursor oligosaccharide under conditions which prevent the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB, to thereby produce an hmGCB preparation.

In a preferred embodiment, the GCB is human GCB. In a preferred embodiment, the cell is a human cell.

In a preferred embodiment, the removal of: one or more α 1.2 mannose residue(s) distal to the pentasaccharide core is prevented; an α 1.3 mannose residue distal to the pentasaccharide core is prevented; and/or an α 1.6 mannose residue distal to the pentasaccharide core is prevented. Preferably, the removal of one or more α 1.2 mannose residue(s) distal to the pentasaccharide core is prevented.

In a preferred embodiment, the method can include contacting the cell with a substance which prevents the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB, e.g., prevents removal of one or more α 1.2-mannose residue(s) distal to the pentasaccharide core, an α 1.3 mannose residue distal to the pentasaccharide core and/or an α 1.6 mannose residue distal to the pentasaccharide core. Preferably, the removal of one or more α 1.2 mannose(s) residue distal to the pentasaccharide core is prevented.

In a preferred embodiment, the method includes contacting the cell with a substance which prevents the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB, wherein the substance is a mannosidase inhibitor. The mannosidase inhibitor can be a class 1 processing mannosidase inhibitor, a class 2 processing mannosidase inhibitor or both. The class 1 processing mannosidase inhibitor can be one or more of: kifunensine, deoxymannojirimycin, or a similar inhibitor. Preferably, the class 1 processing mannosidase inhibitor is kifunensine. Useful class 2 processing mannosidase inhibitors can include one or more of: swainsonine, mannostatin, 6-deoxy-1,4-dideoxy-1,4-imino-D-mannitol (6-deoxy-DIM), and 6-deoxy-6-fluoro-1,4-dideoxy-1,4-imino-D-mannitol (6-deoxy-6-fluoro-DIM). Preferably, the class 2 processing mannosidase inhibitor is swainsonine.

In a preferred embodiment, a mannosidase inhibitor is present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml.

In a preferred embodiment, the method further includes contacting the cell with a class 1 processing mannosidase inhibitor and a class 2 processing mannosidase inhibitor. In a preferred embodiment, the class 1 processing mannosidase inhibitor is present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; the class 2 processing mannosidase inhibitor is present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; each of the class 1 processing and class 2 processing mannosidase inhibitors are present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; the total concentration of the class 1 processing and class 2 processing mannosidase inhibitors

present is between about 0.025 to 40.0 µg/ml, 0.05 to 20 µg/ml, 0.05 to 10 µg/ml, preferably between about 0.1 to 4.0 µg/ml.

In a preferred embodiment, the cell carries a mutation for, e.g., a knockout for, at least one Golgi processing mannosidase. The mutation can be one which reduces the expression of the gene, reduces protein or activity levels, or alters the distribution or other post translational modifications of the mannosidase, e.g., the processing of the carbohydrate chains. The mutation can be one which reduces the level of the Golgi processing mannosidase activity, e.g., one which reduces gene expression, e.g., a null mutation, e.g., a deletion, a frameshift or an insertion. In a preferred embodiment the mutation is a knockout, e.g., in the mannosidase gene. The mutation can affect the structure (and activity of the protein), and can, e.g., be a temperature sensitive mutation or a truncation. In a preferred embodiment, the cell carries a mutation, e.g., a knockout, for: a class 1 processing mannosidase; a class 2 processing mannosidase; a class 1 processing mannosidase and a class 2 processing mannosidase. In a preferred embodiment, the class 1 processing mannosidase is: Golgi mannosidase IA; Golgi mannosidase IB; Golgi mannosidase IC; or combinations thereof. In a preferred embodiment, the class 2 processing mannosidase is: Golgi mannosidase II.

In a preferred embodiment, the cell includes a nucleic acid sequence, such as an antisense molecule or ribozyme, which can bind to or inactivate a cellular mannosidase nucleic acid sequence, e.g., mRNA, and inhibit expression of the protein. In a preferred embodiment, the nucleic acid sequence is: a class 1 processing mannosidase antisense molecule; a class 2 processing mannosidase antisense molecule; both a class 1 processing mannosidase antisense molecule and a class 2 processing mannosidase antisense molecule. In a preferred embodiment, the class 1 processing mannosidase is: Golgi mannosidase IA; Golgi mannosidase IB; Golgi mannosidase IC; and combinations thereof. In a preferred embodiment, the class 2 processing mannosidase is: Golgi mannosidase II.

In a preferred embodiment, the cell includes a molecule, e.g., an exogenously supplied molecule, which binds and inhibits a mannosidase. The molecule can be, e.g., a single chain antibody, an intracellular protein or a competitive or non-competitive inhibitor.

In a preferred embodiment, the hmGCB molecule includes a carbohydrate chain having at least four mannose residues. For example, the molecule has at least one carbohydrate chain having five mannose residues, the hmGCB molecule has at least one carbohydrate chain having six mannose residues, the hmGCB molecule has at least one carbohydrate chain having seven mannose residues, the hmGCB molecule has at least one carbohydrate chain having eight mannose residues, the hmGCB molecule has at least one carbohydrate chain having nine mannose residues. Preferably, the hmGCB molecule has at least one carbohydrate chain having five, eight or nine mannose residues.

In a preferred embodiment, the hmGCB produced (either one or more hmGCB molecules or the preparation as a whole) has a ratio of mannose residues to GlcNAc residues which is greater than 3 mannose residues to 2 GlcNAc residues, preferably the ratio of mannose to GlcNAc is 4:2, 5:2, 6:2, 7:2, 8:2, 9:2; more preferably the ratio of mannose to GlcNAc is 5:2, 8:2 or 9:2.

In a preferred embodiment, the removal of one or more mannose residues distal to the pentasaccharide core is prevented on one, two, three or four of the carbohydrate chains of an hmGCB molecule.

In a preferred embodiment, at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or all of the hmGCB molecules of the preparation have at least one, and preferably two, three or four carbohydrate chains in which the removal of one or more mannose residues distal to the pentasaccharide core has been prevented.

In a preferred embodiment, the hmGCB preparation is a relatively heterogeneous preparation. Preferably, less than 80%, 70%, 60%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5% or 1% of the carbohydrate chains in the hmGCB preparation have the same number of mannose residues in addition to the pentasaccharide core. For example, the ratio of carbohydrate chains having the same number of mannose residues in addition to the pentasaccharide core to carbohydrate chains having a different number of mannose residues can be about: 60%:40%; 50%:50%; 40%:60%; 30%:70%; 25%:75%; 20%:80%; 15%:85%; 10%:90%; 5% or less:95% or more.

In a preferred embodiment, activity of Golgi mannosidase IA and/or IB and/or IC is inhibited and at least about 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or 100% of the carbohydrate chains in the hmGCB preparation have five or more mannose residues, e.g., five, six, seven, eight and/or nine mannose residues. In a preferred embodiment, activity of Golgi mannosidase I is inhibited and the ratio of carbohydrate chains having five or more mannose residues to carbohydrate chains having four or less mannose residues is about 60%:40%; 70%:30%; 75%:25%; 80%:20%; 85%:15%; 90%:10%; 95%:5%; 99%:1% or 100%:0%.

In a preferred embodiment, activity of Golgi mannosidase II is inhibited and at least about 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or 100% of the carbohydrate chains in the hmGCB preparation have five or more mannose residues, e.g., five, six, seven, eight and/or nine mannose residues. In a preferred embodiment, activity of Golgi mannosidase II is inhibited and the ratio of carbohydrate chains having five or more mannose residues to carbohydrate chains having four or less mannose residues is about 60%:40%; 70%:30%; 75%:25%; 80%:20%; 85%:15%; 90%:10%; 95%:5%; 99%:1% or 100%:0%.

In a preferred embodiment, the cell includes an exogenous nucleic acid sequence which includes a GCB coding region. In a preferred embodiment, the cell further includes a regulatory sequence, an endogenous or exogenous regulatory sequence, which functions to regulate expression of the exogenous GCB coding region.

In a preferred embodiment, the cell includes an exogenous regulatory sequence which functions to regulate expression of an endogenous GCB coding sequence, e.g., the regulatory sequence is integrated into the genome of the cell such that it regulates expression of an endogenous GCB coding sequence.

In a preferred embodiment, the regulatory sequence includes one or more of: a promoter, an enhancer, an upstream activating sequence (UAS), a scaffold-attachment region or a transcription factor-binding site. In a preferred embodiment, the regulatory sequence includes: a regulatory sequence from a metallothionein-I gene, e.g., a mouse metallothionein-I gene, a regulatory sequence from an SV-40 gene, a regulatory sequence from a cytomegalovirus gene, a regulatory sequence from a collagen gene, a regulatory sequence from an actin gene, a regulatory sequence from an immunoglobulin gene, a regulatory sequence from the HMG-CoA reductase gene, or a regulatory sequence from the EF-1 α gene.

In a preferred embodiment, the cell is: a eukaryotic cell. In a preferred embodiment, the cell is of fungal, plant or

animal origin, e.g., vertebrate origin. In a preferred embodiment, the cell is: a mammalian cell, e.g., a primary or secondary mammalian cell, e.g., a fibroblast, a hematopoietic stem cell, a myoblast, a keratinocyte, an epithelial cell, an endothelial cell, a glial cell, a neural cell, a cell comprising a formed element of the blood, a muscle cell and precursors of these somatic cells; a transformed or immortalized cell line. Preferably, the cell is a human cell. Examples of immortalized human cell lines useful in the present method include, but are not limited to: a Bowes Melanoma cell (ATCC Accession No. CRL 9607), a Daudi cell (ATCC Accession No. CCL 213), a HeLa cell and a derivative of a HeLa cell (ATCC Accession Nos. CCL2 CCL2.1 and CCL 2.2), a HIL-60 cell (ATCC Accession No. CCL 240), an HT-1080 cell (ATCC Accession No. CCL 121), a Jurkat cell (ATCC Accession No. TIB 152), a KB carcinoma cell (ATCC Accession No. CCL 17), a K-562 leukemia cell (ATCC Accession No. CCL 243), a MCF-7 breast cancer cell (ATCC Accession No. BTH 22), a MOLT-4 cell (ATCC Accession No. 1582), a Namalwa cell (ATCC Accession No. CRL 1432), a Raji cell (ATCC Accession No. CCL 86), a RPMI 8226 cell (ATCC Accession No. CCL 155), a U-937 cell (ATCC Accession No. 1593), WI-28VA13 sub line 2R4 cells (ATCC Accession No. CCL 155), a CCRF-CEM cell (ATCC Accession No. CCL 119) and a 2780AD ovarian carcinoma cell (Van Der Blick et al., Cancer Res. 48:5927-5932, 1988), as well as hetero-hybridoma cells produced by fusion of human cells and cells of another species. In another embodiment, the immortalized cell line can be cell line other than a human cell line, e.g., a CHO cell line, a COS cell line. In another embodiment, the cell can be a from a clonal cell strain or clonal cell line.

In a preferred embodiment, a population of cells which are capable of expressing hmGCB is provided, and at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or all of the cells produce hmGCB molecules with at least one carbohydrate chain, and preferably two, three, or four carbohydrate chains, having the specified number of mannose residues.

In a preferred embodiment, the cell is cultured in culture medium which includes at least one mannosidase inhibitor. In a preferred embodiment, the method further includes obtaining the hmGCB from the medium in which the cell is cultured.

In another aspect, the invention features a method of producing a preparation of hmGCB. The method includes: providing a cell which is capable of expressing GCB; and allowing production of GCB having a precursor oligosaccharide under conditions which inhibit class 1 processing mannosidase activity and class 2 processing mannosidase activity such that the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB is prevented, to thereby produce an hmGCB preparation.

In a preferred embodiment, the GCB is human GCB. In a preferred embodiment, the cell is a human cell.

In a preferred embodiment, the removal of: one or more α 1,2 mannose residue(s) distal to the pentasaccharide core is prevented; an α 1,3 mannose residue distal to the pentasaccharide core is prevented; and/or an α 1,6 mannose residue distal to the pentasaccharide core is prevented. Preferably, the removal of one or more α 1,2 mannose residue(s) distal to the pentasaccharide core is prevented.

In a preferred embodiment, the method can include: contacting the cell with a substance which inhibits a class 1 processing mannosidase activity and a substance which inhibits a class 2 processing mannosidase activity thereby

preventing the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB. In a preferred embodiment, the substances prevent removal of one or more α 1,2 mannose residue distal to the pentasaccharide core.

In a preferred embodiment, the method includes contacting the cell with a substance which inhibits a class 1 processing mannosidase activity and a substance which inhibits a class 2 processing mannosidase activity, wherein the substances are a class 1 processing mannosidase inhibitor and a class 2 processing mannosidase inhibitor. In a preferred embodiment, the class 1 processing mannosidase inhibitor can be one or more of: kifunensine, deoxymannojirimycin, or a similar inhibitor. Preferably, the class 1 processing mannosidase inhibitor is kifunensine. In a preferred embodiment, the class 2 processing mannosidase inhibitor can be one or more of: swainsonine, mannosatin, 6-deoxy-DIM, and 6-deoxy-6-fluoro-DIM. Preferably, the class 2 processing mannosidase inhibitor is swainsonine.

In a preferred embodiment, a class 1 processing mannosidase inhibitor is present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; a class 2 processing mannosidase inhibitor is present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; each of the class 1 processing and class 2 processing mannosidase inhibitors are present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; the total concentration of the class 1 processing and class 2 processing mannosidase inhibitors present is between about 0.025 to 40.0 μ g/ml, 0.05 to 20 μ g/ml, 0.05 to 10 μ g/ml, preferably between about 0.1 to 4.0 μ g/ml.

In a preferred embodiment, the cell carries a mutation for, e.g., a knockout for, a class 1 mannosidase and a class 2 mannosidase. The mutation can be one which reduces the expression of the gene, reduces protein or activity levels, or alters the distribution or other post translational modifications of the mannosidase, e.g., the processing of the carbohydrate chains. The mutation can be one which reduces the level of a class 1 processing mannosidase and/or a class 2 processing mannosidase activity, e.g., one which reduces gene expression, e.g., a null mutation, e.g., a deletion, a frameshift, or an insertion. In a preferred embodiment, the mutation is a knockout in the mannosidase gene. The mutation can affect the structure (and activity of the protein), and can, e.g., be a temperature sensitive mutant. In a preferred embodiment, the class 1 processing mannosidase is: Golgi mannosidase IA; Golgi mannosidase IB; Golgi mannosidase IC; combinations thereof. In a preferred embodiment, the class 2 processing mannosidase is: Golgi mannosidase II.

In a preferred embodiment, the cell includes both a class 1 processing mannosidase antisense molecule and a class 2 processing mannosidase antisense molecule. In a preferred embodiment, the class 1 processing mannosidase is: Golgi mannosidase IA; Golgi mannosidase IB; Golgi mannosidase IC; combinations thereof. In a preferred embodiment, the class 2 processing mannosidase is: Golgi mannosidase II.

In a preferred embodiment, the cell includes a molecule, e.g., an exogenously supplied molecule, which binds and inhibits a mannosidase. The molecule can be, e.g., a single chain antibody, an intracellular protein or a competitive or non-competitive inhibitor.

In a preferred embodiment, the class 1 processing mannosidase activity and the class 2 mannosidase activity can be

inhibited by different mechanisms. For example, a class 1 processing mannosidase activity can be inhibited by contacting the cell with a substrate which inhibits a class 1 processing mannosidase, e.g., a class 1 mannosidase inhibitor, and the class 2 processing mannosidase can be inhibited by using a cell which is a knockout of a class 2 mannosidase and/or includes a class 2 mannosidase antisense molecule. In another preferred embodiment, a class 2 processing mannosidase activity can be inhibited by contacting the cell with a substrate which inhibits a class 2 processing mannosidase, e.g., a class 2 mannosidase inhibitor, and the class 1 processing mannosidase can be inhibited by using a cell which is a knockout of a class 1 mannosidase and/or includes a class 1 mannosidase antisense molecule.

In a preferred embodiment, the hmGCB molecule includes a carbohydrate chain having at least four mannose residues. For example, the hmGCB molecule has at least one carbohydrate chain having five mannose residues, the hmGCB molecule has at least one carbohydrate chain having six mannose residues, the hmGCB molecule has at least one carbohydrate chain having seven mannose residues, the hmGCB molecule has at least one carbohydrate chain having eight mannose residues, the hmGCB molecule has at least one carbohydrate chain having nine mannose residues. Preferably, the hmGCB molecule has at least one carbohydrate chain having five, eight or nine mannose residues.

In a preferred embodiment, the hmGCB produced (either one or more hmGCB molecules or the preparation as a whole) has a ratio of mannose residues to GlcNAc residues which is greater than 3 mannose residues to 2 GlcNAc residues, preferably the ratio of mannose to GlcNAc is 4:2, 5:2, 6:2, 7:2, 8:2, 9:2, more preferably the ratio of mannose to GlcNAc is 8:2 or 9:2.

In a preferred embodiment, the removal of one or more mannose residues distal to the pentasaccharide core is prevented on one, two, three or four of the carbohydrate chains of the hmGCB molecule.

In a preferred embodiment, at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or all of the hmGCB molecules of the preparation have at least one, and preferably two, three or four carbohydrate chains in which the removal of one or more mannose residues distal to the pentasaccharide core has been prevented.

In a preferred embodiment, the hmGCB preparation is a relatively heterogeneous preparation. Preferably, less than 80%, 70%, 60%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5% or 1% of the carbohydrate chains in the hmGCB preparation have the same number of mannose residues in addition to the pentasaccharide core. For example, the ratio of carbohydrate chains having the same number of mannose residues in addition to the pentasaccharide core to carbohydrate chains having a different number of mannose residues can be about: 60%:40%; 50%:50%; 40%:60%; 30%:70%; 25%:75%; 20%:80%; 15%:85%; 10%:90%; 5% or less:95% or more.

In a preferred embodiment, activity of a class 1 processing mannosidase, e.g., Golgi mannosidase IA and/or Golgi mannosidase IB and/or Golgi mannosidase IC, and activity of a class 2 processing mannosidase, e.g., Golgi mannosidase II, are inhibited and at least about 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or 100% of the carbohydrate chains in the hmGCB preparation have five or more mannose residues, e.g., five, six, seven, eight and/or nine mannose residues. In a preferred embodiment, activity of a class 1 processing mannosidase, e.g., Golgi mannosidase IA and/or Golgi mannosidase IB and/or Golgi mannosidase IC, and activity of a class 2 processing mannosidase, e.g., Golgi

mannosidase II, are inhibited and the ratio of carbohydrate chains having five or more mannose residues to carbohydrate chains having four or less mannose residues, respectively, is about 60%:40%; 70%:30%; 75%:25%; 80%:20%; 85%:15%; 90%:10%; 95%:5%; 99%:1% or 100%:0%.

In a preferred embodiment, the cell includes an exogenous nucleic acid sequence which includes a GCB coding region. In a preferred embodiment, the cell further includes a regulatory sequence, an endogenous or exogenous regulatory sequence, which functions to regulate expression of the exogenous GCB coding region.

In a preferred embodiment, the cell includes an exogenous regulatory sequence which functions to regulate expression of an endogenous GCB coding sequence, e.g., the regulatory sequence is integrated into the genome of the cell such that it regulates expression of an endogenous GCB coding sequence.

In a preferred embodiment, the regulatory sequence includes one or more of: a promoter, an enhancer, an upstream activating sequence (UAS), a scaffold-attachment region or a transcription factor-binding site. In a preferred embodiment, the regulatory sequence includes: a regulatory sequence from a metallothionein-I gene, e.g., a mouse metallothionein-I gene, a regulatory sequence from an SV-40 gene, a regulatory sequence from a cytomegalovirus gene, a regulatory sequence from a collagen gene, a regulatory sequence from an actin gene, a regulatory sequence from an immunoglobulin gene, a regulatory sequence from the HMG-CoA reductase gene, or a regulatory sequence from the EF-1 α gene.

In a preferred embodiment, the cell is: a eukaryotic cell. In a preferred embodiment, the cell is of fungal, plant or animal origin, e.g., vertebrate origin. In a preferred embodiment, the cell is: a mammalian cell, e.g., a primary or secondary mammalian cell, e.g., a fibroblast, a hematopoietic stem cell, a myoblast, a keratinocyte, an epithelial cell, an endothelial cell, a glial cell, a neural cell, a cell comprising a formed element of the blood, a muscle cell and precursors of these somatic cells; a transformed or immortalized cell line. Preferably, the cell is a human cell. Examples of immortalized human cell lines useful in the present method include, but are not limited to: a Bowes Melanoma cell (ATCC Accession No. CRL 9607), a Daudi cell (ATCC Accession No. CCL 213), a HeLa cell and a derivative of a HeLa cell (ATCC Accession Nos. CCL2 CCL2.1 and CCL 2.2), a HL-60 cell (ATCC Accession No. CCL 240), an HT-1080 cell (ATCC Accession No. CCL 121), a Jurkat cell (ATCC Accession No. TIB 152), a KB carcinoma cell (ATCC Accession No. CCL 17), a K-562 leukemia cell (ATCC Accession No. CCL 243), a MCF-7 breast cancer cell (ATCC Accession No. BTH 22), a MOLT-4 cell (ATCC Accession No. 1582), a Namalwa cell (ATCC Accession No. CRL 1432), a Raji cell (ATCC Accession No. CCL 86), a RPMI 8226 cell (ATCC Accession No. CCL 155), a U-937 cell (ATCC Accession No. 1593), WI-28VA13 sub line 2R4 cells (ATCC Accession No. CLL 155), a CCRF-CEM cell (ATCC Accession No. CCL 119) and a 2780AD ovarian carcinoma cell (Van Der Blick et al., Cancer Res. 48:5927-5932, 1988), as well as hetero-hybridoma cells produced by fusion of human cells and cells of another species. In another embodiment, the immortalized cell line can be cell line other than a human cell line, e.g., a CHO cell line, a COS cell line. In another embodiment, the cell can be from a clonal cell strain or clonal cell line.

In a preferred embodiment, a population of cells which are capable of expressing hmGCB is provided, and at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%.

98%, 99% or all of the cells produce hmGCB with at least one carbohydrate chain, preferably two, three, or four carbohydrate chains, having the specified number of mannose residues.

In a preferred embodiment, the cell is cultured in a culture medium which includes at least one class 1 processing mannosidase inhibitor and at least one class 2 processing mannosidase inhibitor. In a preferred embodiment, the method further includes obtaining the hmGCB from the medium in which the cell is cultured.

In another aspect, the invention features a method of producing a preparation of hmGCB. The method includes: providing a cell into which a nucleic acid sequence comprising an exogenous regulatory sequence has been introduced such that the regulatory sequence regulates the expression of an endogenous GCB coding region; and

allowing production of GCB having a precursor oligosaccharide under conditions which prevent the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB, to thereby produce an hmGCB preparation.

In a preferred embodiment, the GCB is human GCB.

In a preferred embodiment, the removal of: one or more α 1,2 mannose residue(s) distal to the pentasaccharide core is prevented; an α 1,3 mannose residue distal to the pentasaccharide core is prevented; and/or an α 1,6 mannose residue distal to the pentasaccharide core is prevented. Preferably, the removal of one or more α 1,2 mannose residue(s) distal to the pentasaccharide core is prevented.

In a preferred embodiment, the method can include contacting the cell with a substance which prevents the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB, e.g., prevents removal of one or more α 1,2 mannose residue(s) distal to the pentasaccharide core, an α 1,3 mannose residue distal to the pentasaccharide core and/or an α 1,6 mannose residue distal to the pentasaccharide core. Preferably, the removal of one or more α 1,2 mannose(s) residue distal to the pentasaccharide core is prevented.

In a preferred embodiment, the method includes contacting the cell with a substance which prevents the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB, and the substance is a mannosidase inhibitor. The mannosidase inhibitor can be a class 1 processing mannosidase inhibitor, a class 2 processing mannosidase inhibitor or both. The class 1 processing mannosidase inhibitor can be one or more of: kifunensine, deoxymannojirimycin, or a similar inhibitor. Preferably, the class 1 processing mannosidase inhibitor is kifunensine. Useful class 2 processing mannosidase inhibitors can include one or more of: swainsonine, mannosidase, 6-deoxy-DIM, 6-deoxy-6-fluoro-DIM. Preferably, the class 2 processing mannosidase inhibitor is swainsonine.

In a preferred embodiment, a mannosidase inhibitor is present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml.

In a preferred embodiment, the method further includes contacting the cell with a class 1 processing mannosidase inhibitor and a class 2 processing mannosidase inhibitor. In a preferred embodiment, a class 1 processing mannosidase inhibitor is present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; a class 2 processing mannosidase inhibitor is present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; each of the class

1 processing and class 2 processing mannosidase inhibitors are present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; the total concentration of the class 1 processing and class 2 processing mannosidase inhibitors present is between about 0.025 to 40.0 μ g/ml, 0.05 to 20 μ g/ml, 0.05 to 10 μ g/ml, preferably between about 0.1 to 4.0 μ g/ml.

In a preferred embodiment, the cell carries a mutation for, e.g., a knockout for, at least one mannosidase. The mutation can be one which reduces the expression the gene, reduces protein or activity levels, or alters the distribution or other post translational modifications of the mannosidase, e.g., the processing of the carbohydrate chains. The mutation can be one which reduces the level of the Golgi processing mannosidase activity, e.g., one which reduces gene expression, e.g., a null mutation, e.g., a deletion, a frameshift or an insertion. In a preferred embodiment the mutation is a knockout, e.g., in the mannosidase gene. The mutation can affect the structure (and activity of the protein), and can, e.g., be a temperature sensitive mutation or a truncation. In a preferred embodiment, the cell carries a mutation, e.g., a knockout, for a class 1 processing mannosidase: a class 2 processing mannosidase; a mutant, e.g., a knockout, for a class 1 processing mannosidase and a class 2 processing mannosidase. In a preferred embodiment, the class 1 processing mannosidase is: Golgi mannosidase IA; Golgi mannosidase IB; Golgi mannosidase IC; or combinations thereof. In a preferred embodiment, the class 2 processing mannosidase is: Golgi mannosidase II.

In a preferred embodiment, the cell includes a nucleic acid sequence, such as an antisense molecule or ribozyme, which can bind to or inactivate a cellular mannosidase nucleic acid sequence, e.g., mRNA, and inhibit expression of the protein.

In a preferred embodiment, the nucleic acid sequence is: a class 1 processing mannosidase antisense molecule; a class 2 processing mannosidase antisense molecule; both a class 1 processing mannosidase antisense molecule and a class 2 processing mannosidase antisense molecule. In a preferred embodiment, the class 1 processing mannosidase is: Golgi mannosidase IA; Golgi mannosidase IB; Golgi mannosidase IC; combinations thereof. In a preferred embodiment, the class 2 processing mannosidase is: Golgi mannosidase II.

In a preferred embodiment, the cell includes a molecule, e.g., an exogenously supplied molecule, which binds and inhibits a mannosidase. The molecule can be, e.g., a single chain antibody, an intracellular protein or a competitive or non-competitive inhibitor.

In a preferred embodiment, the hmGCB molecule includes a carbohydrate chain having at least four mannose residues. For example, the hmGCB molecule has at least one carbohydrate chain having five mannose residues, the hmGCB molecule has at least one carbohydrate chain having six mannose residues, the hmGCB molecule has at least one carbohydrate chain having seven mannose residues, the hmGCB molecule has at least one carbohydrate chain having eight mannose residues, the hmGCB molecule has at least one carbohydrate chain having nine mannose residues. Preferably, the hmGCB molecule has at least one carbohydrate chain having five, eight or nine mannose residues.

In a preferred embodiment, the hmGCB produced (either one or more hmGCB molecules or the preparation as a whole) has a ratio of mannose residues to GlcNAc residues which is greater than 3 mannose residues to 2 GlcNAc residues, preferably the ratio of mannose to GlcNAc is 4:2, 5:2, 6:2, 7:2, 8:2, 9:2, more preferably the ratio of mannose to GlcNAc is 5:2, 8:2 or 9:2.

In a preferred embodiment, the removal of one or more mannose residues distal to the pentasaccharide core is prevented on one, two, three or four of the carbohydrate chains of the hmGCB molecule.

In a preferred embodiment, at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or all of the hmGCB molecules of the preparation have at least one, and preferably two, three or four carbohydrate chains in which the removal of one or more mannose residues distal to the pentasaccharide core has been prevented.

In a preferred embodiment, the hmGCB preparation is a relatively heterogeneous preparation. Preferably, less than 80%, 70%, 60%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5% or 1% of the carbohydrate chains in the hmGCB preparation have the same number of mannose residues in addition to the pentasaccharide core. For example, the ratio of carbohydrate chains having the same number of mannose residues in addition to the pentasaccharide core to carbohydrate chains having a different number of mannose residues can be about: 60%:40%; 50%:50%; 40%:60%; 30%:70%; 25%:75%; 20%:80%; 15%:85%; 10%:90%; 5% or less:95% or more.

In a preferred embodiment, activity of Golgi mannosidase IA and/or IB and/or IC is inhibited and at least about 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or 100% of the carbohydrate chains in the hmGCB preparation have five or more mannose residues, e.g., five, six, seven, eight, and/or nine mannose residues. In a preferred embodiment, activity of Golgi mannosidase I is inhibited and the ratio of carbohydrate chains having five or more mannose residues to carbohydrate chains having four or less mannose residues is about 60%:40%; 70%:30%; 75%:25%; 80%:20%; 85%:15%; 90%:10%; 95%:5%; 99%:1%; or 100%:0%.

In a preferred embodiment, activity of Golgi mannosidase II is inhibited and at least about 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or 100% of the carbohydrate chains in the hmGCB preparation have five or more mannose residues. In a preferred embodiment, activity of Golgi mannosidase II is inhibited and the ratio of carbohydrate chains having five or more mannose residues to carbohydrate chains having four or less mannose residues is about 60%:40%; 70%:30%; 75%:25%; 80%:20%; 85%:15%; 90%:10%; 95%:5%; 99%:1%; or 100%:0%.

In a preferred embodiment, the regulatory sequence includes one or more of: a promoter, an enhancer, an upstream activating sequence (UAS), a scaffold-attachment region or a transcription factor-binding site. In a preferred embodiment, the regulatory sequence includes: a regulatory sequence from a metallothionein-I gene, e.g., a mouse metallothionein-I gene, a regulatory sequence from an SV-40 gene, a regulatory sequence from a cytomegalovirus gene, a regulatory sequence from a collagen gene, a regulatory sequence from an actin gene, a regulatory sequence from an immunoglobulin gene, a regulatory sequence from the HMG-CoA reductase gene, or a regulatory sequence from the EF-1 α gene.

In a preferred embodiment, the cell is: a eukaryotic cell. In a preferred embodiment, the cell is of fungal, plant or animal origin, e.g., vertebrate origin. In a preferred embodiment, the cell is: a mammalian cell, e.g., a primary or secondary mammalian cell, e.g., a fibroblast, a hematopoietic stem cell, a myoblast, a keratinocyte, an epithelial cell, an endothelial cell, a glial cell, a neural cell, a cell comprising a formed element of the blood, a muscle cell and precursors of these somatic cells; a transformed or immortalized cell line. Preferably, the cell is a human cell. Examples of immortalized human cell lines useful in the

present method include, but are not limited to: a Bowes Melanoma cell (ATCC Accession No. CRL 9607), a Daudi cell (ATCC Accession No. CCL 213), a HeLa cell and a derivative of a HeLa cell (ATCC Accession Nos. CCL2, CCL2.1 and CCL 2.2), a HL-60 cell (ATCC Accession No. CCL 240), an HT-1080 cell (ATCC Accession No. CCL 121), a Jurkat cell (ATCC Accession No. TIB 152), a KB carcinoma cell (ATCC Accession No. CCL 17), a K-562 leukemia cell (ATCC Accession No. CCL 243), a MCF-7 breast cancer cell (ATCC Accession No. B1H 22), a MOLT-4 cell (ATCC Accession No. 1582), a Namalwa cell (ATCC Accession No. CRL 1432), a Raji cell (ATCC Accession No. CCL 86), a RPMI 8226 cell (ATCC Accession No. CCL 155), a U-937 cell (ATCC Accession No. 1593), WI-28VA13 sub line 2R4 cells (ATCC Accession No. CCL 155), a CCRF-CEM cell (ATCC Accession No. CCL 119) and a 2780AD ovarian carcinoma cell (Van Der Blick et al., Cancer Res. 48:5927-5932, 1988), as well as heterohybridoma cells produced by fusion of human cells and cells of another species. In another embodiment, the immortalized cell line can be cell line other than a human cell line, e.g., a CHO cell line, a COS cell line. In another embodiment, the cell can be from a clonal cell strain or clonal cell line.

In a preferred embodiment, a population of cells which are capable of expressing hmGCB is provided, and at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or all of the cells produce hmGCB with at least one carbohydrate chain, preferably two, three, or four carbohydrate chains, having the specified number of mannose residues.

In a preferred embodiment, the cell is cultured in culture medium which includes at least one mannosidase inhibitor. In a preferred embodiment, the method further includes obtaining the hmGCB from the medium in which the cell is cultured.

In another aspect, the invention features an hmGCB molecule, e.g., an hmGCB molecule described herein, e.g., a human hmGCB, produced by any of the methods described herein. Preferably, the hmGCB molecule includes at least one carbohydrate chain, preferably two, three, or four carbohydrate chains, having at least four mannose residues of a precursor oligosaccharide chain.

In another aspect, the invention features an hmGCB preparation which includes a portion of hmGCB molecules which include at least one carbohydrate chain, preferably two, three, or four carbohydrate chains, having at least four mannose residues of a precursor oligosaccharide chain. Preferably, the hmGCB preparation is produced by any of the methods described herein.

In a preferred embodiment, the hmGCB is human hmGCB.

In a preferred embodiment, the hmGCB molecule can have: at least one carbohydrate chain having five mannose residues; at least one carbohydrate chain having six mannose residues; at least one carbohydrate chain having seven mannose residues; at least one carbohydrate chain having eight mannose residues; at least one carbohydrate chain having nine mannose residues.

In a preferred embodiment, the hmGCB produced (either one or more hmGCB molecules or the preparation as a whole) has at least one carbohydrate chain having a ratio of mannose residues to GlcNAc residues which is greater than 3 mannose residues to 2 GlcNAc residues, preferably the ratio of mannose to GlcNAc is 4:2, 5:2, 6:2, 7:2, 8:2, 9:2, more preferably the ratio of mannose to GlcNAc is 5:2, 8:2 or 9:2.

In a preferred embodiment, at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or all of the hmGCB of the preparation have at least one, preferably, two, three or four carbohydrate chains in which the removal of one or more mannose residues distal to the pentasaccharide core has been prevented.

In another aspect, the invention features a cell having at least one mannosidase activity inhibited and which includes a nucleic acid sequence comprising an exogenous regulatory sequence which has been introduced such that the regulatory sequence regulates the expression of an endogenous GCB coding region, wherein the cell produces GCB in which the removal of at least one mannose residue distal to the pentasaccharide core of a precursor oligosaccharide of GCB is prevented.

In a preferred embodiment, the cell produces an hmGCB preparation, e.g., a human hmGCB preparation, in which the removal of: one or more α 1,2 mannose residue(s) distal to the pentasaccharide core is prevented; an α 1,3 mannose residue distal to the pentasaccharide core is prevented; and/or an α 1,6 mannose residue distal to the pentasaccharide core is prevented. Preferably, the removal of one or more α 1,2 mannose residue(s) distal to the pentasaccharide core is prevented.

In a preferred embodiment, at least one mannosidase activity in the cell has been inhibited by contacting the cell with a substance which inhibits a mannosidase. In a preferred embodiment, the substance is a mannosidase inhibitor. The mannosidase inhibitor can be a class 1 processing mannosidase inhibitor, a class 2 processing mannosidase inhibitor or both. In a preferred embodiment, the class 1 processing mannosidase inhibitor can be one or more of: kifunensine and deoxymannojirimycin. Preferably, the class 1 processing mannosidase inhibitor is kifunensine. In a preferred embodiment, the class 2 processing mannosidase inhibitor can be one or more of: swainsonine, mannosidase, 6-deoxy-DIM, and 6-deoxy-6-fluoro-DIM. Preferably, the class 2 processing mannosidase inhibitor is swainsonine.

In a preferred embodiment, the cell carries a mutation for, e.g., a knockout for, at least one Golgi processing mannosidase. The mutation can be one which reduces the expression of the gene, reduces protein or activity levels, or alters the distribution or other post translational modifications of the mannosidase, e.g., the processing of a carbohydrate chain. The mutant can be one which reduces the level of Golgi processing mannosidase activity, e.g., one which reduces gene expression, e.g., a null mutation, e.g., a deletion, a frameshift, or an insertion. In a preferred embodiment, the mutation is a knockout in the mannosidase gene. The mutation can affect the structure (and activity of the protein), and can, e.g., be a temperature sensitive mutation. In a preferred embodiment, the cell is a mutant, e.g., a knockout, for: a class 1 processing mannosidase; a class 2 processing mannosidase; a class 1 processing mannosidase and a class 2 processing mannosidase. In a preferred embodiment, the class 1 processing mannosidase is: Golgi mannosidase IA; Golgi mannosidase IB; Golgi mannosidase IC; combinations thereof. In a preferred embodiment, the class 2 processing mannosidase is: Golgi mannosidase II.

In a preferred embodiment, the cell further includes a nucleic acid sequence, such as an antisense molecule or ribozyme, which can bind to or inactivate a cellular mannosidase nucleic acid sequence, e.g., mRNA, and inhibit expression of the protein. In a preferred embodiment, the nucleic acid sequence is: a class 1 processing mannosidase antisense molecule; a class 2 processing mannosidase antisense molecule; both a class 1 processing mannosidase

antisense molecule and a class 2 processing mannosidase antisense molecule. In a preferred embodiment, the class 1 processing mannosidase is: Golgi mannosidase IA; Golgi mannosidase IB; Golgi mannosidase IC; combinations thereof. In a preferred embodiment, the class 2 processing mannosidase is: Golgi mannosidase II.

In a preferred embodiment, the cell includes a molecule, e.g., an exogenously supplied molecule, which binds and inhibits a mannosidase. The molecule can be, e.g., a single chain antibody, an intracellular protein or a competitive or non-competitive inhibitor.

In a preferred embodiment, the hmGCB molecule produced by the cell has a ratio of mannose residues to GlcNAc residues which is greater than 3 mannose residues to 2 GlcNAc residues, preferably the ratio of mannose to GlcNAc is 4:2, 5:2, 6:2, 7:2, 8:2, 9:2, more preferably the ratio of mannose to GlcNAc is 5:2, 8:2 or 9:2.

In a preferred embodiment, the cell is unable to remove of one or more mannose residues distal to the pentasaccharide core on one, two, three or four of the carbohydrate chains of hmGCB.

In a preferred embodiment, at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or all of the hmGCB molecules produced by the cell have at least one, preferably, two, three or four carbohydrate chains in which the removal of one or more mannose residues distal to the pentasaccharide core has been prevented.

In a preferred embodiment, the regulatory sequence includes one or more of: a promoter, an enhancer, an upstream activating sequence (UAS), a scaffold-attachment region or a transcription factor-binding site. In a preferred embodiment, the regulatory sequence includes: a regulatory sequence from a metallothionein-I gene, e.g., a mouse metallothionein-I gene, a regulatory sequence from an SV-40 gene, a regulatory sequence from a cytomegalovirus gene, a regulatory sequence from a collagen gene, a regulatory sequence from an actin gene, a regulatory sequence from an immunoglobulin gene, a regulatory sequence from the HMG-CoA reductase gene, or a regulatory sequence from the EF-1 α gene.

In a preferred embodiment, the cell is: a eukaryotic cell. In a preferred embodiment, the cell is of fungal, plant or animal origin, e.g., vertebrate origin. In a preferred embodiment, the cell is: a mammalian cell, e.g., a primary or secondary mammalian cell, e.g., a fibroblast, a hematopoietic stem cell, a myoblast, a keratinocyte, an epithelial cell, an endothelial cell, a glial cell, a neural cell, a cell comprising a formed element of the blood, a muscle cell and precursors of these somatic cells; a transformed or immortalized cell line. Preferably, the cell is a human cell. Examples of immortalized human cell lines useful in the present method include, but are not limited to: a Bowes Melanoma cell (ATCC Accession No. CRL 9607), a Daudi cell (ATCC Accession No. CCL 213), a HeLa cell and a derivative of a HeLa cell (ATCC Accession Nos. CCL2, CCL2.1 and CCL 2.2), a HL-60 cell (ATCC Accession No. CCL 240), an HT-1080 cell (ATCC Accession No. CCL 121), a Jurkat cell (ATCC Accession No. TIB 152), a KB carcinoma cell (ATCC Accession No. CCL 17), a K-562 leukemia cell (ATCC Accession No. CCL 243), a MCF-7 breast cancer cell (ATCC Accession No. BTH 22), a MOLT-4 cell (ATCC Accession No. 1582), a Namalwa cell (ATCC Accession No. CRL 1432), a Raji cell (ATCC Accession No. CCL 86), a RPMI 8226 cell (ATCC Accession No. CCL 155), a U-937 cell (ATCC Accession No. 1593), WI-28VA13 sub line 2R4 cells (ATCC Accession No. CLL 155), a CCRF-CEM cell (ATCC Accession No. CCL

119) and a 2780AD ovarian carcinoma cell (Van Der Blick et al., Cancer Res. 48:5927-5932, 1988), as well as hetero-hybridoma cells produced by fusion of human cells and cells of another species. In another embodiment, the immortalized cell line can be cell line other than a human cell line, e.g., a CHO cell line, a COS cell line. In another embodiment, the cell can be from a clonal cell strain or clonal cell line.

In another aspect, the invention features a pharmaceutical composition which includes an hmGCB molecule, e.g., a human hmGCB, which includes at least one carbohydrate chain, preferably two, three, or four carbohydrate chains, having at least four mannose residues of a precursor oligosaccharide chain, in an amount suitable for treating Gaucher disease.

In a preferred embodiment, the pharmaceutical composition further includes a pharmaceutically acceptable carrier or diluent.

Another aspect of the invention features a method of treating a subject having Gaucher disease. The method includes administering to a subject having Gaucher disease an hmGCB preparation, e.g., a human hmGCB preparation, which includes at least one carbohydrate chain, preferably two, three, or four carbohydrate chains, having at least four mannose residues of a precursor oligosaccharide chain, in an amount suitable for treating Gaucher disease.

In another aspect, the invention features a method of purifying hmGCB from a sample. The method includes: providing a harvested hmGCB product; and subjecting the hmGCB product to hydrophobic charge induction chromatography (HCIC) and/or hydrophobic interaction chromatography (HIC), thereby obtaining purified hmGCB.

In a preferred embodiment, chromatography material MEP HYPERCEL® is used for HCIC. In another preferred embodiment, chromatography material MACROPREP METHYL® is used for HIC.

In another preferred embodiment, the method further includes subjecting the hmGCB product to ion exchange chromatography. The hmGCB product can be subjected to HCIC and/or HIC prior to ion exchange chromatography or the hmGCB product can be subjected to ion exchange chromatography prior to HCIC and/or HIC. Preferably, the hmGCB product is subjected to more than one ion exchange chromatography step. The ion exchange chromatography can be: anion exchange chromatography, cation exchange chromatography or both.

In a preferred embodiment, anion exchange chromatography is performed using one or more of the following chromatography materials: Q SEPHAROSE FAST FLOW®, MACROPREP HIGH Q SUPPORT®, DEAE SEPHAROSE FAST FLOW®, AND MACRO-PREP DEAE®. In a preferred embodiment, cation exchange chromatography is performed using one or more of: SP Sepharose Fast Flow®, SOURCE 30S®, CM Sepharose Fast Flow®, Macro-Prep CM Support®, and Macro-Prep High S Support®.

In a preferred embodiment, the method further includes subjecting the hmGCB product to size exclusion chromatography. Preferably, the size exclusion chromatography is performed using one or more of the following chromatography materials: SUPERDEX 200®, SEPHACRYL S-200 HR® AND BIO-GEL A 1.5M®.

In another aspect, the invention features a method of purifying hmGCB. The method includes: providing a harvested hmGCB product; subjecting the hmGCB product to hydrophobic charge induction chromatography (HCIC) and/or hydrophobic interaction chromatography (HIC); and subjecting the hmGCB product to one or more of anion

exchange chromatography, cation exchange chromatography, and size exclusion chromatography; to thereby obtain purified hmGCB.

In a preferred embodiment, chromatography material MEP HYPERCEL® is used for HCIC. In another preferred embodiment, chromatography material MACROPREP METHYL® is used for HIC.

In a preferred embodiment, the method includes using anion exchange chromatography. Preferably, anion exchange chromatography is performed using one or more of the following chromatography materials: Q SEPHAROSE FAST FLOW®, MACROPREP HIGH Q SUPPORT®, DEAE SEPHAROSE FAST FLOW®, AND MACRO-PREP DEAE®.

In a preferred embodiment, the method includes using cation exchange chromatography. Preferably, cation exchange chromatography is performed using one or more of the following chromatography materials: SP SEPHAROSE FAST FLOW®, SOURCE 30S®, CM SEPHAROSE FAST FLOW®, MACRO-PREP CM SUPPORT®, AND MACRO-PREP HIGH S SUPPORT®.

In a preferred embodiment, the method includes using size exclusion chromatography. Preferably, the size exclusion chromatography is performed using one or more of the following chromatography materials: SUPERDEX 200®, SEPHACRYL S-200 HR® AND BIO-GEL A 1.5M®.

In a preferred embodiment, the hmGCB is subjected to (in any order): anion exchange chromatography and cation exchange chromatography; anion exchange chromatography and size exclusion chromatography; cation exchange chromatography and size exclusion chromatography; anion exchange chromatography, cation exchange chromatography and size exclusion chromatography. Preferably, the hmGCB is subjected to all three of these chromatography steps in the following order: anion exchange chromatography, cation exchange chromatography and size exclusion chromatography.

In another aspect, the invention features a method of purifying hmGCB. The method includes: providing a harvested hmGCB product; subjecting the hmGCB product to hydrophobic charge induction chromatography (HCIC) and/or hydrophobic interaction chromatography (HIC); subjecting the HCIC and/or HIC purified hmGCB product to anion exchange chromatography; subjecting the anion exchange purified hmGCB to cation exchange chromatography; and, subjecting the cation exchange purified hmGCB to size exclusion chromatography, to thereby obtain purified hmGCB.

In a preferred embodiment, chromatography material MEP HYPERCEL® is used for HCIC. In another preferred embodiment, chromatography material MACROPREP METHYL® is used for HIC.

In a preferred embodiment, anion exchange chromatography is performed using one or more of the following chromatography materials: Q Sepharose Fast Flow®, MacroPrep High Q Support®, DEAE Sepharose Fast Flow®, and Macro-Prep DEAE®.

In a preferred embodiment, cation exchange chromatography is performed using one or more of the following chromatography materials: SP SEPHAROSE FAST FLOW®, SOURCE 30S®, CM SEPHAROSE FAST FLOW®, MACRO-PREP CM SUPPORT®, AND MACRO-PREP HIGH S SUPPORT®.

In a preferred embodiment, size exclusion chromatography is performed using one or more of the following chromatography materials: SUPERDEX 200®, SEPHACRYL S-200 HR® AND BIO-GEL A 1.5M®.

The term "high mannose glucocerebrosidase (hmGCB)" as used herein refers to glucocerebrosidase having at least one carbohydrate chain having four or more mannose residues from a precursor oligosaccharide. Preferably, the hmGCB has five, six, seven, eight or nine mannose residues from the precursor oligosaccharide chain. Most preferably, the hmGCB has five, eight or nine mannose residues from the precursor oligosaccharide chain.

The term "hmGCB preparation" refers to two or more hmGCB molecules.

The term "primary cell" includes cells present in a suspension of cells isolated from a vertebrate tissue source (prior to their being plated i.e., attached to a tissue culture substrate such as a dish or flask), cells present in an explant derived from tissue, both of the previous types of cells plated for the first time, and cell suspensions derived from these plated cells. The term secondary cell or cell strain refers to cells at all subsequent steps in culturing. That is, the first time a plated primary cell is removed from the culture substrate and replated (passaged), it is referred to herein as a secondary cell, as are all cells in subsequent passages. Secondary cells are cell strains which consist of secondary cells which have been passaged one or more times. A cell strain consists of secondary cells that: 1) have been passaged one or more times; 2) exhibit a finite number of mean population doublings in culture; 3) exhibit the properties of contact-inhibited, anchorage dependent growth (anchorage-dependence does not apply to cells that are propagated in suspension culture); and 4) are not immortalized. A "clonal cell strain" is defined as a cell strain that is derived from a single founder cell. A "heterogenous cell strain" is defined as a cell strain that is derived from two or more founder cells.

"Immortalized cells", as used herein, are cell lines (as opposed to cell strains with the designation "strain" reserved for primary and secondary cells), a critical feature of which is that they exhibit an apparently unlimited lifespan in culture.

The term "transfected cell" refers to a cell into which an exogenous synthetic nucleic acid sequence, e.g., a sequence which encodes a protein, is introduced. Once in the cell, the synthetic nucleic acid sequence can integrate into the recipient's cells chromosomal DNA or can exist episomally. Standard transfection methods can be used to introduce the synthetic nucleic acid sequence into a cell, e.g., transfection mediated by liposome, polybrene, DEAE dextran-mediated transfection, electroporation, calcium phosphate precipitation or microinjection. The term "transfection" does not include delivery of DNA or RNA into a cell by a virus.

The term "infected cell" or "transduced cell" refers to a cell into which an exogenous synthetic nucleic acid sequence, e.g., a sequence which encodes a protein, is introduced by a virus. Viruses known to be useful for gene transfer include an adenovirus, an adeno-associated virus, a herpes virus, a mumps virus, a poliovirus, a retrovirus, a Sindbis virus, a lentivirus and a vaccinia virus such as a canary pox virus.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

BRIEF DESCRIPTION OF THE DRAWING

FIG. 1 is a diagram showing the trimming of N-linked glycans as it occurs in the endoplasmic reticulum, the intermediate compartment and in the Golgi apparatus. The enzymes are numbered as follows: (1) α -glucosidase I; (2) α -glucosidase II; (3) ER mannosidase I; (4) ER mannosidase

II; (5) ER glucosyl transferase; (6) endomannosidase; (7) Golgi mannosidase IA, IB and IC; (8) GlcNAc transferase I; (9) Golgi mannosidase II. Δ : Glucose; \square : GlcNAc; \bullet : Mannose. Enzymes (3) and (7) are inhibited by kifunensine; enzyme (9) is inhibited by swainsonine.

DETAILED DESCRIPTION OF THE INVENTION

The invention is based, in part, on the discovery that inhibition of the removal of one or more mannose residues distal from the pentasaccharide core of a precursor oligosaccharide chain of glucocerebrosidase (GCB), results in high mannose glucocerebrosidase (hmGCB) that is efficiently targeted to mannose receptors. The removal of a mannose residue from the pentasaccharide core of a precursor oligosaccharide chain can be prevented by inhibiting or reducing the activity of one or more mannosidase enzymes, e.g., one or more class I processing mannosidase(s) and/or class 2 processing mannosidase(s). By preventing or inhibiting the removal of one or more mannose residues, hmGCB having at least one carbohydrate chain with four or more mannose residues from the precursor oligosaccharide chain can be obtained.

Gaucher disease is caused by a deficiency of GCB. GCB is required for degradation of glycosphingolipid glucocerebroside. In the absence of GCB, the glucocerebroside accumulates primarily in phagocytic cells, e.g., macrophages, and, ultimately, builds up in the liver, spleen and bone marrow.

Macrophages have mannose receptors. These receptors play a role in receptor-mediated endocytosis by these cells. hmGCB efficiently targets the mannose receptors on macrophages and improves the uptake of GCB (in the form of hmGCB) into these cells. By directing GCB (in the form of hmGCB) to the cells in which glucocerebroside accumulates, hmGCB can be used to hydrolyze glucocerebroside in the macrophages, thereby reducing the subsequent accumulation of this glycolipid in the liver, spleen and bone marrow of patients having Gaucher disease.

Glucocerebrosidase

Nucleotide sequence information is available for genes encoding glucocerebrosidase from various species. (See Horowitz et al. (1989) *Genomics* 4(1):87-96, disclosing the gene sequence (SEQ ID NO: 1) and amino acid sequence (SEQ ID NO:2) of human glucocerebrosidase; Beutler et al. (1992) *Genomics* 12(4):795-800).

Mature human GCB has five potential N-linked glycosylation sites at Asn-19, Asn-59, Asn-146, Asn-270, and Asn-462. Glycosylation occurs at four of the five sites in human tissue derived GCB (Erickson et al. (1985) *J. Biol. Chem.* 260:14319-14324). Studies employing site-directed mutagenesis have demonstrated that the site at Asn-462 is never occupied (Berg-Fussman et al. (1993) *J. Biol. Chem.* 268:14861-14866). Approximately 20% of the released glycan chains from human placental GCB were shown to be of the high mannose type containing up to seven mannose-residues, whereas the majority of the glycan chains were of the complex type with sialylated biantennary and triantennary structures. (Takasaki et al. (1984) *J. Biol. Chem.* 259:10112-10117)

The first event in GCB N-glycosylation is the co-translational transfer in the lumen of the endoplasmic reticulum (ER) of Glc₃Man₅GlcNAc₂ from oligosaccharide-PP-dolichol to nascent peptide. The presence of the three glucose residues on the donor oligosaccharide allows for efficient

transfer to an acceptor-asparagine by oligosaccharyl transferase. Following N-glycosylation, the glucose-residues are rapidly removed from GCB during the folding process by ER glucosidases I and II. Two different ER mannosidases are each capable of hydrolyzing a single mannose residue from $\text{Man}_5\text{GlcNAc}_2$ to form two different isomers of $\text{Man}_4\text{GlcNAc}_2$ (see FIG. 1). Accessible glycans are then further processed in the Golgi to $\text{Man}_3\text{GlcNAc}_2$ by the removal of up to four $\alpha 1,2$ -linked mannose residues by Golgi mannosidase I. There are at least three different human genes encoding related Golgi mannosidase I isoforms (IA, IB, and IC) with slightly different substrate specificities and tissue expression but all are capable of trimming four mannose residues from $\text{Man}_5\text{GlcNAc}_2$ glycans to form $\text{Man}_3\text{GlcNAc}_2$ (Tremblay et al. (Jul. 27, 2000) *J. Biol. Chem.* [cpub ahead of print]). They are located on chromosomes 6q22, 1p13, and 1p35-36 and their cDNA sequences are obtainable from GenBank as X74837, AF027156, and AF261655, respectively.

The final stage of processing that commits a glycan to the biosynthetic pathway for complex glycans requires the initial conversion of $\text{Man}_3\text{GlcNAc}_2$ to $\text{GlcNAcMan}_3\text{GlcNAc}_2$ by the action of GlcNAc transferase I, after which Golgi mannosidase II can catalyze the removal of two further mannose residues to yield $\text{GlcNAcMan}_2\text{GlcNAc}_2$. This is the substrate for glycan elongation by glycosyl transferases located in the trans Golgi and the trans Golgi network to form complex type chains.

If the high mannose chains transferred to GCB in the initial N-glycosylation step can be prevented from being processed to complex chains in the Golgi, then GCB with high mannose chains (hmGCB) will effectively target the mannose receptors on reticuloendothelial cells.

Cells

Primary and secondary cells to be transfected or infected can be obtained from a variety of tissues and include cell types which can be maintained and propagated in culture. For example, primary and secondary cells which can be transfected or infected include fibroblasts, keratinocytes, epithelial cells (e.g., mammary epithelial cells, intestinal epithelial cells), endothelial cells, glial cells, neural cells, formed elements of the blood (e.g., lymphocytes, bone marrow cells), muscle cells and precursors of these somatic cell types. Primary cells are preferably obtained from the individual to whom the transfected or infected primary or secondary cells are administered (i.e., an autologous cell). However, primary cells may be obtained from a donor (other than the recipient) of the same species (i.e., an allogeneic cell) or another species (i.e., a xenogeneic cell) (e.g., mouse, rat, rabbit, cat, dog, pig, cow, bird, sheep, goat, horse, monkey, baboon).

Primary or secondary cells of vertebrate, particularly mammalian, origin can be transfected or infected with an exogenous DNA sequence, e.g., an exogenous DNA sequence encoding a therapeutic protein, and produce an encoded therapeutic protein stably and reproducibly, both in vitro and in vivo, over extended periods of time. In addition, the transfected or infected primary and secondary cells can express the encoded product in vivo at physiologically relevant levels, cells can be recovered after implantation and, upon reculturing, to grow and display their preimplantation properties. Cells can be modified to reduce cell surface histo compatibility complex or foreign carbohydrate moieties to reduce immunogenicity, e.g., a universal donor cell.

Alternatively, primary or secondary cells of vertebrate, particularly mammalian, origin can be transfected or infected with an exogenous DNA sequence which includes a regulatory sequence. Examples of such regulatory sequences include one or more of: a promoter, an UAS, a scaffold attachment region or a transcription binding site. The targeting event can result in the insertion of the regulatory sequence of the DNA sequence, placing a targeted endogenous gene under their control (for example, by insertion of either a promoter or an enhancer, or both, upstream of the endogenous gene or regulatory region). Optionally, the targeting event can simultaneously result in the deletion of an endogenous regulatory sequence, such as the deletion of a tissue-specific negative regular sequence, of a gene. The targeting event can replace an existing regulatory sequence; for example, a tissue-specific enhancer can be replaced by an enhancer that has broader or different cell-type specificity than the endogenous elements, or displays a pattern of regulation or induction that is different from the corresponding nontransfected or noninfected cell. In this regard, the endogenous sequences are deleted and new sequences are added. Alternatively, the endogenous regulatory sequences are not removed or replaced but are disrupted or disabled by the targeting event, such as by targeting the exogenous sequences within the endogenous regulatory elements. Introduction of a regulatory sequence by homologous recombination can result in primary or secondary cells expressing a therapeutic protein which it does not normally express. In addition, targeted introduction of a regulatory sequence can be used for cells which make or contain the therapeutic protein but in lower quantities than normal (in quantities less than the physiologically normal lower level) or in defective form, and for cells which make the therapeutic protein at physiologically normal levels, but are to be augmented or enhanced in their content or production. Methods of activating an endogenous coding sequence are described in U.S. Pat. Nos. 5,641,670, 5,733,761 and 5,968,502, the contents of which are incorporated herein by reference.

The transfected or infected primary or secondary cells may also include a DNA sequence encoding a selectable marker which confers a selectable phenotype upon them, facilitating their identification and isolation. Methods for producing transfected primary or secondary cells which stably express the DNA sequence, clonal cell strains and heterogeneous cell strains of such transfected cells, methods of producing the clonal and heterogeneous cell strains, are known and described, for example, in U.S. Pat. Nos. 5,641,670, 5,733,761 and 5,968,502, the contents of which are incorporated herein by reference.

Transfected primary or secondary cells, can be made by electroporation. Electroporation is carried out at appropriate voltage and capacitance (and corresponding time constant) to result in entry of the DNA construct(s) into the primary or secondary cells. Electroporation can be carried out over a wide range of voltages (e.g., 50 to 2000 volts) and corresponding capacitance. Total DNA of approximately 0.1 to 500 μg is generally used.

Alternatively, known methods such as calcium phosphate precipitation, microinjection, modified calcium phosphate precipitation and polybrene precipitation, liposome fusion and receptor-mediated gene delivery can be used to transfect cells.

Processing of Glucocerebrosidase

Oligosaccharide assembly in cells which have not been treated to prevent removal of mannose residues usually proceeds as discussed below:

The oligosaccharide chains of GCB are attached to the polypeptide backbone by N-glycosidic linkages. N-linked glycans have an amide bond that connects the anomeric carbon (C-1) of a reducing-terminal N-acetylglucosamine (GlcNAc) residue of the oligosaccharide and a nitrogen of an asparagine (Asn) residue of the polypeptide.

Initiation of N-linked oligosaccharide assembly does not occur directly on the Asn residues of the GCB protein, but rather involves preassembly of a lipid-linked 14 sugar precursor oligosaccharide which is then transferred to the protein in the ER during or very soon after its translation from mRNA. A "precursor oligosaccharide" as used herein refers to the oligosaccharide chain involved in the initial steps in biosynthesis of carbohydrate chains. A "precursor oligosaccharide" can be an oligosaccharide structure which includes at least the following sugars: $\text{Man}_5\text{GlcNAc}_2$, for example, a precursor oligosaccharide can have the following structure: $\text{Glc}_1\text{Man}_5\text{GlcNAc}_2$, as shown in FIG. 1. The precursor oligosaccharide is synthesized while attached via a pyrophosphate bridge to a polyisoprenoid carrier lipid, a dolichol. This assembly involves at least six distinct membrane bound glycosyltransferases. Some of these enzymes transfer monosaccharides from nucleotide sugars, while others utilize dolichol-linked monosaccharides as sugar donors. After assembly of the lipid-linked precursor is complete, another membrane-bound enzyme transfers it to sterically accessible Asn residues which occur as part of the sequence -Asn-X-Ser/Thr.

Glycosylated Asn residues of newly-synthesized GCB transiently carry $\text{Glc}_1\text{Man}_5\text{GlcNAc}_2$, also referred to herein as an "unprocessed carbohydrate chain".

The processing of N-linked oligosaccharides is accomplished by the sequential action of a number of membrane-bound enzymes and begins immediately after transfer of the precursor oligosaccharide $\text{Glc}_1\text{Man}_5\text{GlcNAc}_2$ to the protein. The terms "processing", "trimming" and "modifying" are used interchangeably herein.

N-linked oligosaccharide processing can be divided into three stages: removal of the three glucose residues, removal of a variable number of mannose residues, and addition of various sugar residues to the resulting trimmed core.

The removal of the glucose residues in the first stage of processing involves removal of all three glucose residues to generate N-linked $\text{Man}_5\text{GlcNAc}_2$. This structure is also referred to herein as: $\text{Man}\alpha 1-2\text{Man}\alpha 1-2\text{Man}\alpha 1-3[\text{Man}\alpha 1-2\text{Man}\alpha 1-3(\text{Man}\alpha 1-2\text{Man}\alpha 1-6)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}]$ (See FIG. 1, structure 9'). Processing normally continues to the second stage with removal of mannose residues.

Four of the mannose residues of the $\text{Man}_5\text{GlcNAc}_2$ moiety are bound by $\alpha 1,2$ linkages. Up to four of these $\alpha 1,2$ -linked mannose residues can be removed by mannosidase IA, IB and IC to generate N-linked $\text{Man}_{5-n}\text{GlcNAc}_2$.

Protein-linked $\text{Man}_5\text{GlcNAc}_2$ can then serve as a substrate for GlcNAc transferase I, which transfers a $\beta 1,2$ -linked GlcNAc residue from UDP-GlcNAc to the core $\alpha 1,3$ -linked mannose residue to form $\text{GlcNAcMan}_5\text{GlcNAc}_2$. Mannosidase II can then complete the trimming phase of the processing pathway by removing two mannose residues to generate a protein-linked oligosaccharide which contains within it a $\text{Man}_3\text{GlcNAc}_2$, the "pentasaccharide core". The structure $\text{GlcNAcMan}_3\text{GlcNAc}_2$ is then a substrate for GlcNAc transferase II, which can transfer a $\beta 1,2$ -linked GlcNAc residue to the $\alpha 1,6$ -linked mannose residue.

After the trimming phase, monosaccharides are sequentially added to the growing oligosaccharide chain by a series of membrane-bound Golgi glycosyltransferases, each of

which is highly specific with respect to the acceptor oligosaccharide, the donor sugar, and the type of linkage formed between the sugars. These can include distinct GlcNAc transferases (producing $\beta 1,2$; $\beta 1,4$; or $\beta 1,6$ linkages); galactosyltransferases (producing $\beta 1,4$; $\beta 1,3$; and $\alpha 1,3$ linkages); sialyltransferases (one producing $\alpha 2,3$ and another, $\alpha 2,6$ linkages); fucosyltransferases (producing $\alpha 1,2$; $\alpha 1,3$; $\alpha 1,4$ or $\alpha 1,6$ linkages); and a growing list of other enzymes responsible for a variety of unusual linkages. The cooperative action of these glycosyltransferases produces a diverse family of structures collectively referred to as "complex" oligosaccharides. These may contain two, three or four outer branches ("antennae") attached to the invariant core pentasaccharide, $\text{Man}_3\text{GlcNAc}_2$. These structures are referred to in terms of the number of their outer branches: biantennary (two branches), triantennary (three branches) or tetraantennary (four branches). The size of these complex glycans can vary.

Processing of High Mannose Glucocerebrosidase

hmGCB can be produced by reducing or preventing cellular carbohydrate modification (i.e., processing) of GCB. Carbohydrate modification can be prevented by allowing production of GCB under conditions which prevent the removal of at least one mannose residue distal to the pentasaccharide core of a precursor oligosaccharide chain of GCB. For example, one or more of the "trimming" stages during the removal of mannose residues from a precursor oligosaccharide can be prevented.

Cellular mannosidases fall into two broad classes: class 1 processing enzymes, which include ER mannosidase I, Golgi mannosidase IA, IB and IC and which hydrolyze $\alpha 1,2$ -linked mannose residues, and require Ca^{2+} for activity; and class 2 processing enzymes, which include ER mannosidase II, Golgi mannosidase II, cytosolic α -mannosidase, and lysosomal α -mannosidase and which have a broader substrate specificity and do not require Ca^{2+} for activity.

The trimming of mannose residues from the precursor oligosaccharide involves at least the following mannosidase enzymes: Golgi mannosidase IA, IB and IC, and Golgi mannosidase II. By inhibiting one or more of these mannosidases during N-linked oligosaccharide assembly in a cell, GCB can be produced which has at least one carbohydrate chain with one or more mannose residues in addition to the pentasaccharide core. For example, inhibition of both ER mannosidase I and Golgi mannosidase I can produce hmGCB with at least one carbohydrate chain (and preferably all chains) having at least eight mannose residues from the precursor oligosaccharide; inhibition of Golgi mannosidase II can produce hmGCB with at least one carbohydrate chain (and preferably all chains) having at least five mannose residues from the precursor oligosaccharide.

Trimming by a mannosidase can be inhibited, for example, by contacting the cell with a substance which prevents the removal of one or more mannose residues from a precursor oligosaccharide of GCB or by producing GCB in a cell which does not produce or produces at deficient levels at least one mannosidase, or in a cell which produces a mutated and/or inactive mannosidase. For example, the cell can be a knockout for at least one mannosidase, can express at least one antisense mannosidase molecule or can be dominant negative for at least one mannosidase.

Substances which Prevent Removal of Mannose Residues

A substance which prevents the removal of one or more mannose residues from a precursor oligosaccharide of GCB can be used to produce an hmGCB preparation. For example, a cell which expresses GCB can be contacted with

a substance which prevents the removal of one or more α 1,2 mannose residues of a precursor oligosaccharide of GCB, and/or removal of an α 1,3 mannose residue of a precursor oligosaccharide of GCB, and/or removal of an α 1,6 mannose residue of a precursor oligosaccharide of GCB. Preferably, the substance is a mannosidase inhibitor, e.g., a class 1 processing mannosidase inhibitor or a class 2 processing mannosidase inhibitor.

Cellular mannosidases fall into two broad classes on the basis of protein sequence homologies (Moremen et al. (1994) *Glycobiology* 4:113-125). These two classes are mechanistically different. Class 1 enzymes, which include ER mannosidase I and Golgi mannosidase I isoforms, have a mass of about 63-73 kDa, hydrolyze α 1,2-linked mannose residues and require Ca^{2+} for activity. Class 1 processing mannosidases can be blocked, for example, by treatment with a substrate mimic, e.g., a pyranose analog of mannose. For example, class 1 processing mannosidases can be blocked by treatment with one or more of the following enzymatic inhibitors: kifunensine, deoxymannojirimycin, or a combination thereof. Class 2 enzymes, which include ER mannosidase I, Golgi mannosidase II, cytosolic α -mannosidase, and lysosomal α -mannosidase, have a greater mass of about 107-136 kDa, do not require Ca^{2+} for activity and have a broader substrate specificity. Class 2 processing mannosidases can be blocked, for example, by treatment with furanose transition state analogues of the mannosylation (Daniels et al. (1994) *GlycoBiol.* 4:551-566). For example, class 2 processing mannosidases can be blocked by treatment with one or more of the following inhibitors: swainsonine, 6-deoxy-DIM, 6-deoxy-6-fluoro-DIM, mannosstatin A, or combinations thereof.

Kifunensine can be used as an inhibitor of the endoplasmic reticulum mannosidase I and/or Golgi mannosidase IA and/or IB and/or IC; deoxymannojirimycin can be used as an inhibitor of ER mannosidase I, ER mannosidase II and/or of Golgi mannosidase IA and/or IB and/or IC; swainsonine can be used as an inhibitor of Golgi mannosidase II; and mannosstatin A can be used as an inhibitor of Golgi mannosidase II.

Use of a mannosidase inhibitor can inhibit the processing of a carbohydrate chain of GCB past a certain stage of mannose residue trimming during oligosaccharide assembly. For example, contacting a cell with kifunensine can inhibit trimming of any, or one, two, three, or four of the mannose residues of a precursor oligosaccharide.

Processing α -mannosidases can be blocked by treatment of cells with one or more of the following enzyme inhibitors: Kifunensine, an inhibitor of the endoplasmic reticulum I and Golgi mannosidase I enzymes (Weng and Spiro (1993) *J. Biol. Chem.* 268:25656-25663; Elbein et al. (1990) *J. Biol. Chem.* 265:15599-15605).

Swainsonine, an inhibitor of the Golgi mannosidase II enzyme (Tulsiani et al. (1982) *J. Biol. Chem.* 257: 7936-7939).

Deoxymannojirimycin, an inhibitor of both endoplasmic reticulum mannosidases I and II and of Golgi mannosidase I (Weng and Spiro (1993) *J. Biol. Chem.* 268: 25656-25663; Tremblay and Herscovics (2000) *J. Biol. Chem.* July 27; [pub ahead of print]).

DIM (1,4-dideoxy-1,4-imino-D-mannitol), an inhibitor of Golgi mannosidase II (Palamarzyk et al. (1985) *Arch. Biochem. Biophys.* 243:35-45).

6-Deoxy-DIM and 6-deoxy-6-fluoro-DIM, inhibitors of Golgi mannosidase II (Winchester et al. (1993) *Biochem J.* 290:743-749).

Mannostatin A, an inhibitor of Golgi mannosidase II (Tropea et al. (1990) *Biochemistry* 29:10062-10069).

Various mannosidase inhibitors can be selected by their ability to penetrate particular cell types as well as by the inhibitory potency of the mannosidase inhibitor. For example, swainsonine is rapidly internalized by cultured fibroblasts in a time- and concentration-dependent manner. Swainsonine is also a potent inhibitor of a class 2 mannosidase, e.g., Golgi mannosidase II. Thus, swainsonine can be used to produce hmGCB in cultured fibroblasts, e.g., hmGCB having at least one carbohydrate chain which has at least four or five mannose residues of the precursor oligosaccharide. In addition, kifunensine is readily taken up by cultured fibroblasts and is a potent inhibitor of class 1 mannosidases, e.g., ER mannosidase I and Golgi mannosidase I. Thus, kifunensine can be used to produce hmGCB in cultured fibroblasts, e.g., hmGCB having at least one carbohydrate chain which has at least four, five, six, seven, eight or nine mannose residues of the precursor oligosaccharide.

Preferably, the mannosidase inhibitor is present at a concentration of 0.025 to 20.0 $\mu\text{g/ml}$, 0.05 to 10 $\mu\text{g/ml}$, 0.05 to 5 $\mu\text{g/ml}$, preferably between about 0.1 to 2.0 $\mu\text{g/ml}$. For example, a class 1 processing mannosidase inhibitor can be present at a concentration between about 0.025 to 20.0 $\mu\text{g/ml}$, 0.05 to 10 $\mu\text{g/ml}$, 0.05 to 5 $\mu\text{g/ml}$, preferably between about 0.1 to 2.0 $\mu\text{g/ml}$; a class 2 processing mannosidase inhibitor can be present at a concentration between about 0.025 to 20.0 $\mu\text{g/ml}$, 0.05 to 10 $\mu\text{g/ml}$, 0.05 to 5 $\mu\text{g/ml}$, preferably between about 0.1 to 2.0 $\mu\text{g/ml}$; each of the class 1 processing and class 2 processing mannosidase inhibitors can be present at a concentration between about 0.025 to 20.0 $\mu\text{g/ml}$, 0.05 to 10 $\mu\text{g/ml}$, 0.05 to 5 $\mu\text{g/ml}$, preferably between about 0.1 to 2.0 $\mu\text{g/ml}$; or the total concentration of the class 1 processing and class 2 processing mannosidase inhibitors present can be between about 0.025 to 40.0 $\mu\text{g/ml}$, 0.05 to 20 $\mu\text{g/ml}$, 0.05 to 10 $\mu\text{g/ml}$, preferably between about 0.1 to 5.0 $\mu\text{g/ml}$.

The cell can be contacted with a mannosidase inhibitor by, for example, culturing the cell on medium which includes at least one mannosidase inhibitor.

Mannosidase Mutant Cell

Mannosidase Knockout Cell

Permanent or regulated inactivation of mannosidase gene expression can be achieved by targeting to a mannosidase locus with a transfected plasmid DNA construct or a synthetic oligonucleotide. The plasmid construct or oligonucleotide can be designed to several forms. These include the following: 1) insertion of selectable marker genes or other sequences within an exon of a mannosidase gene; 2) insertion of exogenous sequences in regulatory regions of non-coding sequence; 3) deletion or replacement of regulatory and/or coding sequences; and, 4) alteration of a protein coding sequence by site specific mutagenesis.

In the case of insertion of a selectable marker gene into coding sequence, it is possible to create an in-frame fusion of an endogenous mannosidase exon with the mannosidase exon engineered to contain, for example, a selectable marker gene. In this way following successful targeting, the endogenous mannosidase gene expresses a fusion mRNA (mannosidase sequence plus selectable marker sequence). Moreover, the fusion mRNA would be unable to produce a functional mannosidase translation product.

In the case of insertion of DNA sequences into regulatory regions, the transcription of a mannosidase gene can be silenced by disrupting the endogenous promoter region or any other regions in the 5' untranslated region (5' UTR) that is needed for transcription. Such regions include, for

example, translational control regions and splice donors of introns. Secondly, a new regulatory sequence can be inserted upstream of the mannosidase gene that would render the mannosidase gene subject to the control of extracellular factors. It would thus be possible to down-regulate or extinguish mannosidase gene expression as desired for optimal hmGCB production. Moreover, a sequence which includes a selectable marker and a promoter can be used to disrupt expression of the endogenous sequence. Finally, all or part of the endogenous mannosidase gene could be deleted by appropriate design of targeting substrates.

In order to create a cell which includes a knockout of at least one chromosomal copy of the human Golgi mannosidase IA, IB or IC gene, the genomic DNA comprising at least the 5' portion of the gene (including regulatory sequences, 5' UTR, coding sequence) is isolated. For example, the GenBank sequence, Accession No.: NM005907 (human), can be used to generate a probe for Golgi mannosidase IA or Accession Nos.: AAF97058 can be used to generate a probe for Golgi mannosidase IB or IC using polymerase chain reaction (PCR). Oligonucleotides for PCR can be designated based upon the GenBank sequence. The resulting probe can hybridize to the single copy Golgi mannosidase IA, IB or IC gene. This probe can then be used to screen a commercially available recombinant phage library (e.g., a library made from human genomic DNA) to isolate a clone comprising all or part of the mannosidase I structural genes. Once a recombinant clone comprising a mannosidase regulatory and/or coding sequence is isolated, specific targeting plasmids designed to achieve the inactivation of mannosidase gene expression can then be constructed. Inactivation of mannosidase activity results from the insertion of exogenous DNA into regulatory or coding sequences to disrupt the translational reading frame. Inactivation of the enzyme can also be the result of disruption of mRNA transcription or mRNA processing, or by deletion of endogenous mannosidase regulatory or coding sequences.

The nucleic acid sequence of other class 1 and class 2 processing mannosidase are also available, for example, in GenBank. Using the methods described above for Golgi mannosidase IA, IB or IC, a knockout cell for other class 1 and/or class 2 processing mannosidases can be produced.

A mannosidase knockout cell can be used, for example, in gene therapy. A knockout cell can be administered to a subject, e.g., a subject having Gaucher disease, such that the cell produces hmGCB in vivo.

Antisense Mannosidase Nucleic Acid Sequences

Nucleic acid molecules which are antisense to a nucleotide encoding a mannosidase, e.g., a class I processing or class 2 processing mannosidase, can be used as an inactivating agent which inhibits expression of a mannosidase. For example, Golgi mannosidase IA, Golgi mannosidase IB, Golgi mannosidase IC, and/or Golgi mannosidase II expression can be inhibited by an antisense nucleic acid molecule. An "antisense" nucleic acid includes a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a mannosidase, e.g., complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. Accordingly, an antisense nucleic acid can form hydrogen bonds with a sense nucleic acid. The antisense nucleic acid can be complementary to an entire mannosidase coding strand, or to only a portion thereof. For example, an antisense nucleic acid molecule which antisense to the "coding region" of the coding strand of a nucleotide sequence encoding a mannosidase can be used.

As the coding strand sequences encoding various mannosidases are disclosed in, for example, Bause (1993) *Eur. J. Biochem.* 217(2):535-540; Gonzalez et al. (1999) *J. Biol. Chem.* 274(30):21375-21386; Misago et al. (1995) *Proc. Natl. Acad. Sci. USA* 92(25): 11766-11770; Tremblay et al. (1998) *Glycobiology* 8(6):585-595, Tremblay et al. (2000) *J. Biol. Chem.* July 27: [epub ahead of print], antisense nucleic acids can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule can comprise sequence complementary to the entire coding region of a mannosidase mRNA, but more preferably is an oligonucleotide which is complementary to only a portion of the coding or noncoding region of a mannosidase mRNA. For example, the antisense oligonucleotide can comprise sequence complementary to the region surrounding the translation start site of a mannosidase mRNA. An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, or more nucleotides in length. An antisense nucleic acid can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (e.g., an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, e.g., phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl)uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylquenosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylquenosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), ybutoxosine, pseudouracil, quosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methyl ester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl)uracil, (acp3)w, and 2,6-diaminopurine. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (i.e., RNA transcribed from the inserted nucleic acid will be of an antisense orientation relative to a target nucleic acid of interest).

Purification of hmGCB

The term "purified" hmGCB, as used herein, refers to hmGCB that is substantially free of cellular material when produced by a cell which expresses GCB. The language "substantially free of cellular material" includes preparations of hmGCB in which the protein is separated from cellular components of the cells in which it is produced. In one embodiment, the language "substantially free of cellular material" includes preparations of hmGCB having less than about 30% (by dry weight) of non-GCB protein (also referred to herein as a "protein impurity" or "contaminating protein"), more preferably less than about 20% of non-GCB protein, still more preferably less than about 10% of non-GCB protein, and most preferably less than about 5%

non-GCB protein. When the hmGCB is obtained (i.e., harvested) from culture media, it is also preferably substantially free of a component of the culture medium, i.e., components of the culture medium represent less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the dry weight of the protein preparation.

Various methods can be used to harvest hmGCB from culture media. The term "harvested hmGCB" as used herein refers to hmGCB obtained from culture media or from a cell. For example, one of the following alternatives can be used to prepare the harvested hmGCB prior to a purification procedure. These can include: 1) filtering the fresh harvest; 2) filtering the fresh harvest and freezing, e.g., at about -20° C. to -80° C., the filtered product until ready for processing (at which time it can be thawed and, optionally, filtered); 3) filtering the fresh harvest, concentrating filtered product (e.g., by about 8 to 10 fold), and then, optionally, filtering again; 4) filtering the fresh harvest, concentrating filtered product (e.g., by about 8 to 10 fold), optionally, filtering again, and then freezing, e.g., at about -20° C. to -80° C., until ready for processing (at which time it can be thawed and, optionally, filtered). Variations of these alternatives can also be performed. For example, when the harvested product or concentrated harvested product is frozen, different harvests can be pooled after thawing and filtered. In addition, for harvested or concentrated harvested product, the product can be held at a cooling temperature, e.g., about 2° C. to 8° C., for short periods of time, e.g., about 1 to 3 days, preferably 1 day, prior to purification. The harvested product held at the cooling temperature can be pooled prior to purification.

When a concentration of harvest is performed, an ultra-filtration membrane with a 5,000 to 50,000 mw cutoff, preferably a 10,000 to 30,000 mw cutoff, can be employed. Filter clarification will typically employ a 1.2 µm/0.5 µm prefilter, followed by a 0.2 µm final filter.

HmGCB can be purified by the following purification techniques. For example, hydrophobic charge induction chromatography (HCIC) can be used to purify the hmGCB preparation. Alternatively, hydrophobic interaction chromatography (HIC) can be used to purify the hmGCB preparation. Both HCIC and HIC are described below.

HCIC or HIC can be used alone or in combination with one or more ion exchange steps. Ion exchange steps that can be used in combination with an HCIC or HIC step (either before or after HCIC or HIC) include the use of anion exchange and/or cation exchange chromatography. Generally known commercially available anion exchange supports used in the purification of proteins bear quaternary ammonium functional groups. Preferred matrices for use in the present process are agarose or cellulose based matrices such as microcrystalline cellulose or cross-linked agaroses. Also particularly preferred are those matrices bearing diethyl aminoethyl, triethyl aminomethyl, or trimethyl aminomethyl functional groups. A particularly preferred anion exchange matrix is trimethyl aminomethyl crosslinked agarose, which is commercially available, e.g., Q-Sepharose Fast Flow® (Pharmacia). Generally known commercially available cation exchange supports that may be used in the purification of proteins bear acidic functionalities, including carboxy and sulfonic acids. Matrices containing the cation functionalities include various forms of celluloses and polystyrene based matrices. For example, weak cation exchangers known in the art include, but are not limited to, Carboxymethyl-Sepharose® and Carboxymethyl-Cellulose®. Strong cation exchangers known in the art include, but are not limited to,

sulfonated polystyrenes (AG 50W®, Bio-Rex 70®), sulfonated celluloses (SP-Sephadex®), and sulfonated Sepharoses (S-Sepharose®). A particularly preferred cation exchange matrix is S-Sepharose Fast Flow® (Pharmacia).

The chromatographic step involving these matrices is most preferably conducted as a column chromatography step or in alternative a batch absorptive technique, which optionally can be performed at a temperature between 25° C. to 40° C. Preferably, a salt is added to a washing or eluting buffer to increase the ionic strength of the buffer. Any of the salts conventionally used may be employed for this purpose as can be readily determined by one skilled in the art, with NaCl being one of the most frequently and conveniently used salts.

A conventional gel filtration step can also be used in combination with the HCIC or HIC chromatography process step. Representative examples of these matrices are polydextrans cross linked with acrylamides, such as composite hydrophilic gels prepared by covalently cross linking allyl dextran with N,N'-methylene bisacrylamide and crosslinked cellulose or agarose gels. Commercially available crosslinked dextran-acrylamides are known under the trade name Sephacryl® and are available from Pharmacia. Commercially available crosslinked dextran-agarose resins are known under the trade name Superdex®, available from Pharmacia. A preferred Superdex® gel is Superdex 200®. Examples of crosslinked cellulose gels are those commercially available cross linking porous cellulose gels, e.g., GLC 300® or GLC 1,000® that are available from Amicon Inc. Silica based resins such as TSK-Gel SW®, available from TosoHaas can be utilized. Polymer based resins such as TSK-Gel PW®, TSK Alpha Series®, Toyopearl HW packings® (copolymerization of ethylene glycol and methyl acrylate polymers) are also available from TosoHaas.

Preferably, HCIC or HIC can be combined with one or more of these ion exchange steps. When a combination of HCIC or HIC and various ion exchange or gel filtration steps are used, they can be performed in any order. For example, as described below a four step procedure can be followed which includes HCIC using hydrophobic charge induction chromatography material MEP HYPERCEL® or HIC using hydrophobic interaction chromatography material Macro-Prep Methyl®, then ion-exchange chromatography resins Q SEPHAROSE FAST FLOW®, SP SEPHAROSE FAST FLOW®, and lastly size-exclusion chromatography resin SUPERDEX 200®. Several of these procedures are set forth in more detail below.

MEP Hypercel Chromatography

MEP (mercaptoethylpyridine) Hypercel® (BioSeptra, Life Technologies) can be used for HCIC. It is a resin consisting of NEP-linked to a regenerated cellulose bead of high porosity (80-100 microns). The functional group (MEP), consisting of a hydrophobic tail and an ionizable head group, is uncharged at neutral pH and can bind certain protein ligands based on hydrophobic interaction at a physiological ionic strength. Elution is accomplished by decreasing pH to 4 to 5, at which MEP is positively charged, and the protein elutes from the column due to electrostatic repulsion. For example, prepared harvest or harvest concentrate can be applied directly to the MEP column equilibrated with 25 mM sodium phosphate, pH 6.8, containing 180 mM sodium chloride and 2 mM DTT. Optionally, the column can then be washed with equilibration buffer containing 25 mM sodium caprylate until the absorbance at 280 nm (A280) stabilizes. The hmGCB can be eluted from the column with 50 mM sodium acetate, 2 mM DTT, pH 4.7, and the peak as monitored at 280 nm can be collected.

MacroPrep Methyl Chromatography

An alternative to MEP Hypercel® is MacroPrep Methyl®, which is a hydrophobic interaction chromatography (HIC) resin. This resin consists of a methyl functional group attached to a bead composition of macroporous copolymerized glycol methacrylate and diethylene glycol dimethacrylate. For example, MacroPrep Methyl® (BioRad) chromatography can be performed as follows. The pH of the harvest or harvest concentrate is adjusted to 5.6, and ammonium sulfate is added to 0.70 M final concentration. The prepared harvest can be applied to the MacroPrep Methyl® column, which has been equilibrated in 0.70 M ammonium sulfate, 10 mM MES, pH 5.6. After application of the load, the column is washed with equilibrated buffer until the A280 returns to baseline. The hmGCB can be eluted with 10 mM MES, pH 5.6. The eluted hmGCB can be ultrafiltered and/or diafiltered in preparation for steps such as an ion exchange step such as Q Sepharose chromatography, SP Sepharose chromatography and/or Superdex 200 Chromatography.

Q Sepharose Chromatography

Q Sepharose Fast Flow® (Amersham Pharmacia) is a relatively strong anion exchange chromatography resin. The functional substituent is a quaternary amine group, which is positively charged over the working pH range of 2 to 12. Proteins with a net negative charge at the working pH will tend to bind to the resin at a relatively low ionic strength and can be eluted at higher ionic strength or lower pH. HmGCB does not bind to Q Sepharose at approximately pH 6 and low ionic strength, but impurities do bind, thereby purifying the sample. For example, the following protocol can be used to purify hmGCB in the sample by Q Sepharose Fast Flow® chromatography. Under appropriate conditions, hmGCB flows through this column, so the product is found in the flowthrough/wash fraction. Sodium phosphate (250 mM, pH 6) is added to the MEP elution pool prepared as described above to a final concentration of 25 mM, and the pH of the pool is adjusted to pH 6 with NaOH (and HCl if necessary). The conductivity is adjusted to 2.5±0.1 mS/cm by dilution with water or by ultrafiltration/diafiltration using 25 mM sodium phosphate, 2 mM DTT, at approximately pH 6. The material is then filtered and applied to a column of Q Sepharose Fast Flow® which has been equilibrated in 25 mM sodium phosphate, 2 mM DTT, pH 6.0. After application of the load, the column is washed with equilibration buffer until the A280 reaches baseline. The flowthrough/wash fraction can then be processed through another column, e.g., SP Sepharose Fast Flow® column, shortly thereafter, e.g., within 24 hours, or frozen and stored at about -20° C. to -80° C. prior to further processing.

Other strong anion exchange resins, such as MacroPrep High Q Support® (BioRad) can be used in place of Q Sepharose. A weaker anion exchange resin such as DEAE Sepharose Fast Flow® (Pharmacia) or MacroPrep DEAE® (BioRad) can also be used. The column is equilibrated in buffer, e.g., 25 mM sodium phosphate, pH 6. The pH of the sample is adjusted to pH 6 and the conductivity is adjusted by dilution or diafiltration to a relatively low ionic strength, which allows impurities to bind to the column and hmGCB to flow through. The sample is applied and the column is washed with equilibration buffer. Impurities are still bound to the column, and can be eluted with application of salt, e.g., sodium chloride or potassium chloride, or application of a lower pH buffer, or a combination of increased salt and lower pH.

The hmGCB can also be allowed to bind the anion exchange column during loading by decreasing the salt

concentration in the load or by running the column at a higher pH, or by a combination of both decreased salt and higher pH.

SP Sepharose Chromatography

SP Sepharose Fast Flow® (Amersham Pharmacia) is a relatively strong cation exchange chromatography resin. The functional substituent is a charged sulfonic acid group, which is negatively charged over a working pH range of 2 to 12. Proteins with a net positive charge at the working pH will tend to bind to the resin at a relatively low ionic strength and can be eluted at higher ionic strength or higher pH. HmGCB binds to SP Sepharose at approximately pH 6 and intermediate ionic strength (e.g., 6.5 mS/cm) and can be eluted at higher ionic strength (e.g., 10.7 mS/cm). Impurity proteins remain bound to SP Sepharose under conditions of hmGCB elution, thereby purifying the hmGCB in the sample. For example, the following protocol can be used to purify hmGCB by SP Sepharose Fast Flow® chromatography. Sodium chloride (2.0 M stock) is added to the Q Sepharose® flowthrough/wash until the conductivity is 6.3 mS/cm. The pH is checked and readjusted to pH 6.0 if necessary. Then, addition of sodium chloride stock is continued until the conductivity is 6.5 mS/cm. The material is filtered and applied to a column of SP Sepharose Fast Flow®, which has been equilibrated with 25 mM sodium phosphate, 44 mM sodium chloride, pH 6.0. After application of the load, the column is washed with equilibration buffer until the baseline is reached and eluted with 25 mM sodium phosphate, 84 mM sodium chloride, pH 6.0. HmGCB is found in the elution fraction.

Another cation exchange resin, e.g., Source 30S® (Pharmacia), CM Sepharose Fast Flow® (Pharmacia), MacroPrep CM Support® (BioRad) or MacroPrep High S Support® (BioRad), can be used as an alternative to SP Sepharose. The hmGCB can bind to the column at approximately pH 6 and low to intermediate ionic strength, such as 4 to 7 mS/cm. A buffer, e.g., 10 mM sodium citrate, pH 6.0, 10 mM MES, pH 6.0, 25 mM sodium phosphate, pH 6.0, or other buffer with adequate buffering capacity at pH 6.0 can be used to equilibrate the column. The ionic strength of the sample is adjusted by dilution or diafiltration to a level which will accommodate binding to the column. The sample is applied to the column and the column is washed after the load to remove unbound material. A salt, e.g., sodium chloride or potassium chloride, can be used to elute the hmGCB from the column. Alternatively, the hmGCB can be eluted from the column with a buffer of higher pH or a combination of higher salt concentration and higher pH.

The hmGCB can also be made to flow through the cation exchange column during loading by increasing the salt concentration in the equilibration buffer and in the sample load, by running the column at a higher pH or by a combination of both increased salt and higher pH.

Superdex 200 Chromatography

Superdex 200 prep grade® (Amersham Pharmacia) is used for size exclusion chromatography of hmGCB, whereby molecules are separated by size, molecular mass, Stokes radius or hydrodynamic volume. Superdex 200 is composed of dextran covalently cross linked to agarose and has a fractionation range of 10,000 to 60,000 molecular weight for globular proteins. For example, the following protocol can be used to purify hmGCB by Superdex 200® chromatography. The SP elution pool is concentrated by ultrafiltration using a 10,000 mw cutoff membrane. The concentrated pool is filtered, then applied to a Superdex 200 prep grade® column which has been equilibrated in 50 mM sodium citrate, pH 6.0. The A280 of the column effluent in

the initial fractions is collected and, for example, an 8 to 16% SDS polyacrylamide gel is run to determine pooling of fractions. Pooling may be decided based on visual inspection of the silver-stained gel.

Other size exclusion chromatography resins such as Sephacryl S-200 HR®, Bio-Gel A 1.5 m®, or Tosoh Haas TSK Gel resins can also be used to purify hmGCB. The buffer used for size exclusion chromatography of hmGCB is 50 mM sodium citrate, pH 6.0. Other buffers can also be used such as 25 mM sodium phosphate, pH 6.0 containing 0.15 M sodium chloride. The pH of the buffer can be between pH 5 and pH 7 and should have sufficient ionic strength to minimize ionic interactions with the column.

Variations of pH, buffer and/or salt concentration in any of the purification protocols described above can be performed by routine methods to achieve the desired purified product.

Assays for Determining Macrophage Uptake and Cellular Targeting of hmGCB

The uptake efficiency of hmGCB by macrophages can be determined by assaying, e.g., protein levels and/or enzyme activity in macrophages. For example, as described in the Examples below and in Dimant et al. (1987) *J. Leukocyte Biol.* 42:485-490, an in vitro assay using a macrophage cell line can be used to determine absolute and mannose receptor specific uptake of hmGCB.

In addition, in vivo comparison of uptake of hmGCB and GCB by liver cells can be determined as described, for example, in Friedman et al. (1999) *Blood* 93:2807-2816. Briefly a mouse model can be injected with hmGCB or GCB, and then sacrificed shortly thereafter. The liver of the animal can then be used to prepare a suspension of liver cells, e.g., parenchymal cells, Kupffer cells, endothelial cells and hepatocytes. The cells can then be separated, identified by morphology and the protein levels and/or enzymatic activity of hmGCB and GCB in the various liver cell types can be determined. Alternatively, immunohistochemical detection may be used to localize hmGCB to a specific cell or cell type in tissue of treated animals.

Pharmaceutical Compositions

High mannose glucocerebrosidase (hmGCB) can be incorporated into pharmaceutical compositions suitable for administration to a subject, e.g., a human. The composition can include a sufficient dosage of hmGCB to treat a subject having Gaucher disease. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, excipients, dispersion media, coatings, antibacterial and antifungal agents, isotonic and adsorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, such media can be used in the compositions of the invention. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, and subcutaneous administration. Preferably, the route of administration is intravenous. Solutions or suspensions used for parenteral application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or

sodium bisulfite; chelating agents such as ethylenediamine-tetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders, e.g., lyophilized preparations, for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL® (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged stability of the injectable compositions can be brought about by including in the composition an agent which delays adsorption, for example, aluminum monostearate, human serum albumin and gelatin.

Sterile injectable solutions can be prepared by incorporating the hmGCB in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying, e.g., lyophilization, which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811.

Treatment of Gaucher Disease

HmGCB, e.g., any hmGCB molecule or preparation described herein, can be used to treat a subject having Gaucher disease. Alternatively, any mannosidase knockout cell described herein, can be introduced into a subject having Gaucher disease to deliver hmGCB to the subject. Various routes of administration and various sites can be used. Once implanted in individual, the knockout cell can produce hmGCB.

Preferably, the knockout cells used will generally be patient-specific genetically engineered cells. It is possible, however, to obtain cells from another individual of the same species or from a different species. Use of such cells might require administration of an immunosuppressant, alteration of histocompatibility antigens, or use of a barrier device to prevent rejection of the implanted cells.

Gaucher disease is an autosomal recessive lysosomal storage disorder characterized by a deficiency in the lysosomal enzyme, glucocerebrosidase (GCB). GCB hydrolyzes the glycolipid glucocerebroside that is formed after degradation of glycosphingolipids in the membranes of white blood cells and red blood cells. If GCB hydrolysis is insufficient then glucocerebroside can accumulate in macrophages (Gaucher cells), causing anemia, thrombocytopenia, organomegaly and major bone problems.

There are several types of Gaucher disease including Gaucher type 1, type 2 and type 3, which can arise due to various mutations in the GCB gene. A "therapeutically effective amount" of hmGCB, i.e., a dosage of hmGCB sufficient to treat Gaucher disease, can be given to a subject having this disorder. The term "treat" as used herein refers to reducing or inhibiting one or more symptoms of Gaucher disease. Symptoms of Gaucher disease type I include: skeletal complications such as bone pain, bone lesions, osteopenia, osteonecrosis, avascular necrosis and pathological fractures; anemia; hepatosplenomegaly; splenic nodules and liver dysfunction; thrombocytopenia; and/or delayed growth and pubertal development. Symptoms of Gaucher disease type II include the symptoms of Gaucher type I as well as neck rigidity, apathy, catatonias, strabismus, increased deep reflex and laryngeal spasm. Symptoms of Gaucher disease type III are similar to Gaucher type II except milder and later in onset.

A therapeutically effective amount of hmGCB can be determined on an individual basis and will be based, at least in part, on consideration of the size of the patient, the agent used, the type of delivery system used, the time of administration relative to the severity of the disease, and whether a single, multiple, or a controlled release dose regimen is employed. Preferably, the dosage of hmGCB sufficient to treat Gaucher disease is less than the dosage of human tissue derived or human placenta derived GCB, or GCB produced by cells in vitro and then trimmed to expose core mannose residues.

Treatment of Other Lysosomal Storage Diseases

Generally, the invention described herein can be used to produce proteins for targeting any cells that express mannose receptors on their surface. Thus, the invention described herein can be used to treat any disorder in which it is desirable to target a protein for treatment to a mannose receptor-expressing cell. For example, the invention described herein can also be applied to other lysosomal storage enzymes and other lysosomal storage diseases in which cells, e.g., the cells of reticuloendothelial origin, accumulate undigested substrate. Reticuloendothelial cells include macrophages, Kupfer cells in the liver and histio-

cytes in the spleen. Such lysosomal storage diseases include, but are not limited to, Farber disease and Neimann-Pick disease.

Farber disease is an autosomal recessive lysosomal storage disorder characterized by a deficiency in acid ceramidase. Ceramidases are enzymes responsible for degradation of ceramide. If ceramide degradation is insufficient then ceramide accumulates leading to granuloma formation and histiocytic response. (Moser, H. W. Ceramidase deficiency: Farber lipogranulomatosis; In: *The Metabolic and Molecular Basis of Inherited Disease* (C. R. Scriver, A. L. Beaudet, W. S. Sly and D. Valle, Eds.), Seventh edition, pp. 2589-2599, McGraw-Hill Inc., New York (1995))

There are several types of Farber disease including Farber type 1, type 2, type 3, type 4, and type 5 which differ in severity and sites of major tissue involvement. There is also type 6 and type 7 Farber disease. High mannose acid ceramidase can be given to a subject having Farber disease to treat, i.e., alleviate or reduce at least one symptom, of the disease. Symptoms of Farber disease type 1 include: swelling of the joints (particularly the interphalangeal, metacarpal, ankle, wrist, knee and elbow), palpable nodules in relation to the affected joints and over pressure points, a hoarse cry that may progress to aphonia, feeding and respiratory difficulty, poor weight gain and intermittent fever. The symptoms usually occur between ages two weeks and four months. Symptoms of Farber type 2 and type 3 include: subcutaneous nodules, joint deformities, and laryngeal involvement. These subjects survive longer than subjects having Farber type 1. Farber disease type 5 symptoms include psychomotor deterioration beginning at one to two and half years of age.

Neimann-Pick disease type A and type B are an autosomal recessive lysosomal storage disorder characterized by a deficiency acid sphingomyelinase. Acid sphingomyelinase is an enzyme responsible for degradation of sphingomyelin. If sphingomyelinase is deficient, sphingomyelin and other lipids can accumulate in the monocyte-macrophage system. (Schuman, E. H. and Desnick, R. J. Neimann-Pick Disease types A and B: acid sphingomyelinase deficiencies; In: *The Metabolic and Molecular Basis of Inherited Disease* (C. R. Scriver, A. L. Beaudet, W. S. Sly and D. Valle, Eds.), Seventh edition, pp. 2589-2599, McGraw-Hill Inc., New York (1995))

There are several types of Neimann-Pick disease including type A and type B. High mannose acid sphingomyelinase can be given to a subject having Neimann-Pick disease to treat, i.e., alleviate or reduce at least one symptom, of the disease. Symptoms of Neimann-Pick disease type A include: enlargement of the spleen and liver, lymphadenopathy, microcytic anemia, decreased platelet count, hypotonia, muscular weakness, psychomotor retardation. Symptoms of Neimann-Pick type B include: enlargement of the liver and/or spleen, hepatosplenomegaly; pulmonary compromise.

Thus, high mannose lysosomal storage enzymes such as high mannose acid ceramidase or high mannose acid sphingomyelinase can be produced by the methods described herein in order to target these proteins to mannose receptor-expressing cells.

EXAMPLES

In experiments with HT-1080 cells in which the glucocerebrosidase gene has been activated as described in U.S. 5,641,670 (Gene-Activated™ GCB (GA-GCB)), the cells were treated with either kifunensine or swainsonine at concentrations ranging from 0.1 to 2 μ M.

Effect of Kifunensine or Swainsonine on GA-GCB Glycoforms

HT-1080 cells producing GA-GCB were plated in duplicate 6-well plates and the Production Medium adjusted to the following concentrations of kifunensine or swainsonine: 0 (no drug), 0.1, 0.25, 0.5, 1, and 2 $\mu\text{g/mL}$. The medium was harvested and the cells refed every 24 hours for three days. The samples from the third day were subjected to isoelectric focusing (IEF) analysis. The effect of kifunensine and swainsonine on the molecular charge of GA-GCB is shown by the IEF analysis. With both drugs, a concentration dependent increase in the apparent isoelectric point (pI) was observed, with kifunensine causing a much larger shift in pI than swainsonine at the highest concentration tested (2 $\mu\text{g/mL}$).

Effect of Kifunensine or Swainsonine on GA-GCB Production

Ten roller bottles (surface area, 1700 cm^2 each) were seeded in Growth Medium (DMEM with 10% calf serum) with HT-1080 cells producing GA-GCB. Following two weeks of growth, the medium was aspirated and 200 mL of fresh Production Medium (DMEM/F12, 0% calf serum) was added to three sets of roller bottles. Two sets of 4 roller bottles were treated with 1 $\mu\text{g/mL}$ of either kifunensine or swainsonine. The third group of two roller bottles received no drug treatment. After approximately 24 hours, the medium from each roller bottle was harvested, pooled and a sample taken for GA-GCB enzymatic activity analysis. This procedure was repeated for seven days. Stable production of GA-GCB was observed for all roller bottles throughout the seven daily harvests (Table 1). Absolute levels of the enzyme, however, varied according to drug treatment group with the following average GA-GCB production levels observed across the seven harvests: 38.3 ± 3.5 mg/L (control, no drug treatment), 24.5 ± 4.0 mg/L (swainsonine, 1 $\mu\text{g/mL}$), and 21.3 ± 2.8 mg/L (kifunensine, 1 $\mu\text{g/mL}$). Both drugs, therefore, resulted in stable, but lower production levels with the largest decrease seen for kifunensine (44% reduction relative to control).

TABLE 1

Roller Bottle Production of Glucocerebrosidase in Cells Treated with Mannosidase Inhibitors								
Treatment	Glucocerebrosidase ^{a)} Activity (^{b)} mg/Liter							Average \pm Standard Deviation
	Harvest 1	Harvest 2	Harvest 3	Harvest 4	Harvest 5	Harvest 6	Harvest 7	
No drug added	35.8	36.6	44.9	40.5	34.6	38.3	37.2	38.3 ± 3.5
Swainsonine (1 $\mu\text{g/mL}$)	28.6	17.4	28.5	27.0	22.9	25.0	22.3	24.5 ± 4.0
Kifunensine (1 $\mu\text{g/mL}$)	26.0	22.9	17.7	21.2	18.4	21.0	22.0	21.3 ± 2.8

^{a)} Assay performed as follows: test article is mixed with the enzyme substrate (4-methylumbelliferyl- β -D-glucopyranoside) and incubated for 1 hour at 37° C. The reaction is stopped by the addition of NaOH/Glycine buffer. Fluorescence is quantified by the use of a fluorescence spectrophotometer.

^{b)} Specific activity: 2,500 Units/mg. One unit is defined as the conversion of 1 μMole of substrate in 1 hour at 37° C.

Effect of Kifunensine or Swainsonine on GA-GCB Uptake into Macrophages

GA-GCB produced in HT-1080 cells was used in an in vitro assay to determine uptake efficiency in a mouse macrophage cell line. The specific objective of the experiment

was to determine the absolute and mannose receptor-specific uptake of GA-GCB in mouse J774E cells. One day prior to assay, J774E cells were plated at 50,000 cells/ cm^2 in 12 well plates in Growth Medium. For the assay, 0.5 mL of Production Medium (DMEM/F12, 0% calf serum) containing 50 nM vitamin D3 (1,2-5, Dihydroxy vitamin D3) was added to the cells. Unpurified GA-GCB (from harvest 4, Table 1) was added to the test wells at a final concentration of 10 $\mu\text{g/mL}$ in the presence or absence of 2 $\mu\text{g/mL}$ mannan (a competitor for the mannose receptor). Three different forms of GA-GCB were used: GA-GCB from cells treated with kifunensine (1 $\mu\text{g/mL}$), GA-GCB from cells treated with swainsonine (1 $\mu\text{g/mL}$), and GA-GCB (1 $\mu\text{g/mL}$) from untreated cells. Control wells received no GA-GCB. The wells were incubated for 4 hours at 37° C., then washed extensively in buffered saline, scraped into GA-GCB enzyme reaction buffer, passed through 2 freeze/thaw cycles, and clarified by centrifugation. The supernatant was then quantitatively tested for enzyme activity and total protein. Internalization of GA-GCB into mouse J774E cells is shown in Table 2 and is reported as Units/mg of cell lysate. These results demonstrated that uptake of GA-GCB from kifunensine treated cells was 14-fold over background and 73% inhibitable by mannan and that uptake of GA-GCB from swainsonine treated cells was 7-fold over background and 67% inhibitable by mannan. In addition, they also demonstrate that uptake of GA-GCB from untreated cells was approximately 3-fold over background and 53% inhibitable by mannan. Thus, the inhibition of intracellular mannosidases by either kifunensine or swainsonine results in GA-GCB that can be transported into cells efficiently via the mannose receptor, with kifunensine causing an approximately 2-fold greater uptake than swainsonine. Improvement in targeting of GA-GCB to cells via mannose receptors can therefore be optimized by production of GA-GCB in the presence of kifunensine or swainsonine.

TABLE 2

Internalization of Glucocerebrosidase Into J774E Cells. Glucocerebrosidase Produced from Cells Treated with Mannosidase Inhibitors

Sample	Glucocerebrosidase Activity (Units/mg cell lysate)		
	Absolute	Background Corrected	Inhibition (%)
Background (no GA-GCB added)	655	0	—
GA-GCB from untreated cells +	2816	2161	—
Mannan	1678	1023	53
GA-GCB from kifunensine treated cells + Mannan	9185	8530	73
GA-GCB from swainsonine treated cells + Mannan	2977	2322	—
	4787	4132	—
	2036	1381	67

^{a)} Assay performed as follows: sample is mixed with the enzyme substrate (4-methylumbelliferyl- β -D-glucopyranoside) and incubated for 1 hour at 37° C. The reaction is stopped by the addition of NaOH/Glycine buffer. Fluorescence is quantified by the use of a fluorescence spectrophotometer. Total protein determined in freeze/thaw cell lysates by bicinchoninic acid (BCA). Activity reported as units/mg total protein. One Unit is defined as the conversion of 1 μMole of substrate in 1 hour at 37° C.

^{b)} Cells treated with drug received 1 $\mu\text{g/mL}$ of either Kifunensine or Swainsonine in the presence or absence of mannan (2 $\mu\text{g/mL}$).

Purification and Characterization of hmGCB

HmGCB was purified from the culture medium of human fibroblasts grown in the presence of kifunensine at a con-

centration of 2 µg/ml. The four N-linked glycans present on hmGCB were released by peptide N-glycosidase F and purified using a Sep-pak C18 cartridge. Oligosaccharides eluting in the 5% acetic acid fraction were permethylated using sodium hydroxide and methyl iodide, dissolved in methanol:water (80:20), and portions of the permethylated glycan mixture were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF-MS). The sample was analyzed on a Voyager STR Biospectrometry Research Station laser-desorption mass spectrometer coupled with Delayed Extraction using a matrix of 2,5-dihydroxybenzoic acid. A pattern of pseudo-molecular ions is seen in the range m/z 1500–2500, indicating the presence of high-mannose glycans ranging from Man₅GlcNAc₂ to Man₉GlcNAc₂.

TABLE 3

Summary of Data Obtained from MALDI-TOF-MS Analysis of N-Glycans from hmGCB from Kifunensine-Treated Cells

M/Z	Peak Assignment	Approximate % of Total Glycans
1580	Man ₉ GlcNAc ₂	1.3
1730	Man ₈ GlcNAc ₂	11.2
1752		
1784		
1934	Man ₇ GlcNAc ₂	23.3
1957		
1988		
2139	Man ₆ GlcNAc ₂	32.0
2161		
2192		

TABLE 3-continued

Summary of Data Obtained from MALDI-TOF-MS Analysis of N-Glycans from hmGCB from Kifunensine-Treated Cells

M/Z	Peak Assignment	Approximate % of Total Glycans
2343	Man ₅ GlcNAc ₂	31.2
2365		
2397		
2969	Biantennary complex	1.0

The most abundant high mannose glycans present are Man₉GlcNAc₂ and Man₈GlcNAc₂, with decreasing abundances of Man₇GlcNAc₂, Man₆GlcNAc₂, and Man₅GlcNAc₂. A trace amount of a fucosylated biantennary complex glycan containing two sialic acid residues was observed. An approximate indication of the relative abundance of each glycan is obtained by measuring the peak heights. See Table 3. A more accurate assessment of the average chain length of the high mannose glycans was obtained by MALDI-TOF-MS analysis of the intact glycoprotein. A sharp peak was obtained at m/z 62,483.1 due to the homogeneity of the glycan chains. The mass of the mature peptide calculated from the amino acid sequence is 55,577.6, indicating the four N-linked glycan chains contribute 6905.5 to the total mass of hmGCB. From this number, it can be calculated that the average glycan length is 8.15 mannose residues.

All patents and references cited herein are incorporated in their entirety by reference.

Other embodiments are within following claims.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 2

<210> SEQ ID NO 1

<211> LENGTH: 8850

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 1

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What is claimed:

1. A method of producing a preparation of high mannose 60
 glucocerebrosidase (hmGCB) comprising a carbohydrate
 chain having at least four mannose residues, comprising:
 providing a mammalian cell that expresses a human
 glucocerebrosidase (GCB);
 contacting the cell with kifunensine;
 allowing the cell to produce hmGCB; and

harvesting the hmGCB from the cell or its culture media,
 to thereby produce an hmGCB preparation.

2. The method of claim 1, wherein removal of one or more
 α 1,2 mannose residue(s) distal to the pentasaccharide core
 is prevented.

3. The method of claim 1, wherein the kifunensine is
 65 present at a concentration between about 0.05 to 20.0 µg/ml.

4. The method of claim 3, wherein the kifunensine is
 present at a concentration between about 0.1 to 2.0 µg/ml.

5. The method of claim 1, further comprising contacting the cell with a class 2 processing mannosidase inhibitor.
6. The method of claim 5, wherein the class 2 processing mannosidase inhibitor is selected from the group consisting of: swainsonine, mannosatin, 6-deoxy DIM, 6-deoxy-6-fluoro-DIM and combinations thereof.
7. The method of claim 5, wherein the class 2 processing mannosidase inhibitor is swainsonine.
8. The method of claim 5, wherein the class 2 processing mannosidase inhibitor is present at a concentration between 0.05 to 20.0 $\mu\text{g/ml}$.
9. The method of claim 1, wherein the hmGCB has at least one carbohydrate chain having five mannose residues.
10. The method of claim 1, wherein the hmGCB has at least one carbohydrate chain having eight mannose residues.
11. The method of claim 1, wherein the hmGCB has at least one carbohydrate chain having nine mannose residues.
12. The method of claim 1, wherein the removal of one or more mannose residues distal to the pentasaccharide core is prevented on at least two carbohydrate chains of hmGCB.
13. The method of claim 1, wherein at least 60% of the hmGCB of the preparation have one or more carbohydrate chains in which the removal of one or more mannose residues distal to the pentasaccharide core has been prevented.
14. The method of claim 13, wherein the removal of three or more mannose residues distal to the pentasaccharide core has been prevented.
15. The method of claim 1, wherein at least about 20% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.
16. The method of claim 15, wherein at least about 40% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.
17. The method of claim 16, wherein at least about 60% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.
18. The method of claim 1, wherein at least about 80% or more of the carbohydrate chains of the hmGCB preparation have six or more mannose residues.
19. The method of claim 1, wherein the cell is a human cell and is a knockout for a class 2 processing mannosidase.
20. The method of claim 1, wherein the cell is a human cell and comprises a class 2 processing mannosidase antisense molecule.
21. The method of claim 1, wherein the cell comprises an exogenous nucleic acid sequence comprising a GCB coding region.
22. The method of claim 21, wherein the cell further comprises an exogenous regulatory sequence which functions to regulate expression of the GCB coding region.
23. The method of claim 1, wherein the cell comprises an exogenous regulatory sequence which functions to regulate expression of an endogenous GCB coding sequence.
24. The method of claim 1, wherein the cell is a primary cell.
25. The method of claim 1, wherein the cell is a secondary cell.
26. The method of claim 1, wherein the cell is a human cell.
27. The method of claim 26, wherein the cell is a fibroblast or a myoblast.
28. The method of claim 26, wherein the cell is an immortalized cell.
29. The method of claim 27, wherein the cell is an HT-1080 cell.

30. The method of claim 1, wherein the cell is contacted with kifunensine in culture media.
31. The method of claim 30, wherein the hmGCB is obtained from the media in which the cell is cultured.
32. A method of producing a preparation of high mannose glucocerebrosidase (hmGCB) comprising a carbohydrate chain having at least four mannose residues, the method comprising:
 - providing a human cell into which a nucleic acid sequence comprising an exogenous regulatory sequence has been introduced such that the regulatory sequence is operably linked to, and regulates the expression of, an endogenous GCB coding region;
 - contacting the cell with a class 1 mannosidase inhibitor such that the removal of at least one mannose residue distal to the pentasaccharide core of a precursor oligosaccharide of GCB is prevented; and
 - allowing the cell to produce hmGCB, to thereby produce an hmGCB preparation.
33. The method of claim 32, wherein the mannosidase inhibitor prevents the removal of one or more α 1,2 mannose residue(s) distal to the pentasaccharide core.
34. The method of claim 32, wherein the mannosidase inhibitor further prevents the removal of one α 1,3 mannose residue distal to the pentasaccharide core.
35. The method of claim 32, wherein the mannosidase inhibitor further prevents the removal of one α 1,6 mannose residue distal to the pentasaccharide core.
36. The method of claim 32, wherein the class 1 processing mannosidase inhibitor is kifunensine.
37. The method of claim 36, wherein the kifunensine is present at a concentration between about 0.05 to 20.0 $\mu\text{g/ml}$.
38. The method of claim 37, wherein the kifunensine is present at a concentration between about 0.1 to 2.0 $\mu\text{g/ml}$.
39. The method of claim 32, wherein the cell is further contacted with a class 2 mannosidase inhibitor.
40. The method of claim 39, wherein the class 2 processing mannosidase inhibitor is selected from the group consisting of: swainsonine, mannosatin, 6-deoxy DIM, 6-deoxy-6-fluoro-DIM and combinations thereof.
41. The method of claim 39, wherein the class 2 processing mannosidase inhibitor is swainsonine.
42. The method of claim 39, wherein the class 2 processing mannosidase inhibitor is present at a concentration between 0.05 to 20.0 $\mu\text{g/ml}$.
43. The method of claim 32, wherein the cell is a knockout for a class 2 processing mannosidase.
44. The method of claim 32, wherein the cell comprises a class 2 processing mannosidase antisense molecule.
45. The method of claim 32, wherein the hmGCB has at least one carbohydrate chain having six mannose residues of the precursor oligosaccharide.
46. The method of claim 32, wherein the hmGCB has at least one carbohydrate chain having eight mannose residues of the precursor oligosaccharide.
47. The method of claim 32, wherein the hmGCB has at least one carbohydrate chain having nine mannose residues of the precursor oligosaccharide.
48. The method of claim 32, wherein the mannosidase inhibitor prevents removal of at least three mannose residues distal to the pentasaccharide core of the precursor oligosaccharide of GCB.
49. The method of claim 32, wherein the mannosidase inhibitor prevents removal of one or more mannose residues distal to the pentasaccharide core on at least two of the carbohydrate chains of hmGCB.

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50. The method of claim 32, wherein at least 60% of the hmGCB of the preparation have one or more carbohydrate chains in which the removal of three or more mannose residues distal to the pentasaccharide core has been prevented.

51. The method of claim 32, wherein at least 20% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.

52. The method of claim 51, wherein at least 40% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.

53. The method of claim 52, wherein at least 60% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.

54. The method of claim 32, wherein at least about 80% or more of the carbohydrate chains of the hmGCB preparation have six or more mannose residues.

55. The method of claim 32, wherein the cell is a primary cell.

56. The method of claim 32, wherein the cell is a secondary cell.

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57. The method of claim 32, wherein the cell is a fibroblast or a myoblast.

58. The method of claim 32, wherein the cell is an immortalized cell.

59. The method of claim 58, wherein the cell is an HT-1080 cell.

60. The method of claim 36, wherein the cell is contacted with kifunensine in culture media.

61. The method of claim 60, wherein the hmGCB is obtained from the media in which the cell is cultured.

62. The method of claim 1, wherein the cell is a Chinese hamster ovary (CHO) cell transfected with an exogenous nucleic acid sequence comprising a human GCB coding sequence.

63. The method of claim 1, wherein the cell is a COS cell transfected with an exogenous nucleic acid sequence comprising a human GCB coding sequence.

* * * * *

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

**Assignee: Shire Human Genetic Therapies,
Inc.**

**Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS**

Attachment E

Maintenance Fee Statement



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PATENT NUMBER	FEE AMT	SUR CHARGE	PYMT DATE	U.S. APPLICATION NUMBER	PATENT ISSUE DATE	APPL. FILING DATE	PAYMENT YEAR	SMALL ENTITY?	ATTY DKT NUMBER
7,138,262	\$980.00	\$0.00	03/25/10	09/641,471	11/21/06	08/18/00	04	NO	HGT 0013

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment F

Brumshtein et al. (2010) Glycobiology 20(1):24-32

Characterization of gene-activated human acid- β -glucosidase: Crystal structure, glycan composition, and internalization into macrophages

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Received on June 1, 2009; revised on August 30, 2009; accepted on September 1, 2009

Gaucher disease, the most common lysosomal storage disease, can be treated with enzyme replacement therapy (ERT), in which defective acid- β -glucosidase (GlcCerase) is supplemented by a recombinant, active enzyme. The X-ray structures of recombinant GlcCerase produced in Chinese hamster ovary cells (imiglucerase, Cerezyme[®]) and in transgenic carrot cells (prGCD) have been previously solved. We now describe the structure and characteristics of a novel form of GlcCerase under investigation for the treatment of Gaucher disease, Gene-ActivatedTM human GlcCerase (velaglucerase alfa). In contrast to imiglucerase and prGCD, velaglucerase alfa contains the native human enzyme sequence. All three GlcCerasees consist of three domains, with the active site located in domain III. The distances between the carboxylic oxygens of the catalytic residues, E340 and E235, are consistent with distances proposed for acid-base hydrolysis. Kinetic parameters (K_m and V_{max}) of velaglucerase alfa and imiglucerase, as well as their specific activities, are similar. However, analysis of glycosylation patterns shows that velaglucerase alfa displays distinctly different structures from imiglucerase and prGCD. The predominant glycan on velaglucerase alfa is a high-mannose type, with nine mannose units, while imiglucerase contains a chitobiose tri-mannosyl core glycan with fucosylation. These differences in glycosylation affect cellular internalization; the rate of velaglucerase alfa internalization into human macrophages is at least 2-fold greater than that of imiglucerase.

Keywords: Gaucher disease/gene activation/
glucocerebrosidase/glycans/mannose-6-phosphate receptor/
site-specific glycosylation/X-ray structure

Introduction

Gaucher disease is caused by mutations in the gene encoding the lysosomal enzyme, acid- β -glucosidase (glucocerebrosidase, GlcCerase, E.C. 3.2.1.45) (Beutler and Grabowski 2001;

Futerman and Zimran 2006). The most common treatment for Gaucher disease is enzyme replacement therapy (ERT), in which defective GlcCerase is supplemented with an active enzyme. ERT using imiglucerase, a recombinant analog of human GlcCerase expressed in Chinese hamster ovary (CHO) cells has been available for ~15 years. After expression and purification, imiglucerase is modified by exo-glycosidase treatment (Friedman and Hayes 1996) to expose the core mannose residues that can be recognized by macrophages. Glycan remodeling greatly improves targeting to and internalization by macrophages, the main cell type affected in Gaucher disease (Futerman and Zimran 2006). An alternative means of producing GlcCerase (prGCD) in transgenic carrot root cells has been developed (Aviezer et al. 2009). The X-ray structures of imiglucerase and prGCD have been previously reported (Dvir et al. 2003; Shaaltiel et al. 2007).

In the current study, we have used gene activation in a well-characterized, continuous human cell line to produce gene-activated human acid- β -glucocerebrosidase (velaglucerase alfa). Gene activation refers to targeted recombination with a promoter that activates the endogenous GlcCerase gene in the selected human cell line. Velaglucerase alfa is secreted as a monomeric glycoprotein of approximately 63 kDa and is composed of 497 amino acids with a sequence identical to that of the natural human protein (Zimran et al. 2007). Glycosylation of velaglucerase alfa is altered by using kifunensine, a mannosidase I inhibitor, during cell culture, which results in the secretion of a protein containing predominantly high-mannose type glycans (Elbein et al. 1990).

Herein we describe the crystal structure of velaglucerase alfa, using a preparation that had been partially deglycosylated, and show that it is similar to that of imiglucerase (Dvir et al. 2003) and prGCD (Shaaltiel et al. 2007). Velaglucerase alfa differs from imiglucerase and prGCD as the latter two enzymes contain a mutation at residue 495 (an Arg to His substitution: R495H), and prGCD contains seven additional residues at the C terminus (DLLVDTM) and two additional residues at the N terminus (EF). Moreover, the kinetic parameters and specific activity of velaglucerase alfa are very similar to those of imiglucerase. We also compare the glycosylation patterns of velaglucerase alfa and imiglucerase by use of LC-MS and assess the impact of the different glycosylation patterns by analyzing internalization in human macrophages.

Results and discussion

X-ray structure

Diffraction-quality crystals of velaglucerase alfa were obtained after partial deglycosylation using *N*-glycosidase F, by a procedure similar to that previously described for imiglucerase (Dvir et al. 2003). Velaglucerase alfa crystallized in the same space

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Table I. Data collection and refinement statistics

	Velaglucerase alfa
Data collection	
Space group	C222 ₁
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	109.37, 285.55, 91.69
<i>abg</i> (°)	90.00, 90.00, 90.00
Resolution (Å)	19.9–2.7 (2.75–2.70) ^a
<i>R</i> _{sym} (%)	15.7 (51.0)
<i>I</i> / <i>σ</i> (<i>I</i>)	14.4 (4.6)
Completeness (%)	100 (100)
Redundancy	7.5 (7.6)
Refinement	
Resolution (Å)	19.9–2.7
Number of reflections	39,776
<i>R</i> _{work} / <i>R</i> _{free}	17.3/23.4
Rms deviations	
Bond lengths (Å)	0.012
Bond angles (°)	1.486
Number of refined atoms	
Protein	7871
Carbohydrates	70
Ions	90
Solvent	326
Ramachandran outliers (%)	0.4

^aThe highest resolution shell is shown in parentheses.

group, C222₁, as imiglucerase (Table I), and unit cell parameters were similar to the previously published GlcCerase structures (Dvir et al. 2003; Premkumar et al. 2005; Brumshtein et al. 2006). The asymmetric unit contained two copies of velaglucerase alfa, designated as molecules A and B. The root mean square deviation (RMSD) value between molecules A and

B (<0.3 Å) shows that they are virtually identical. A comparison of the structures of imiglucerase, prGCD, and velaglucerase alfa demonstrates that these structures are very similar, with an RMSD of 0.35–0.46 Å (Table II).

Velaglucerase alfa thus consists of three noncontiguous domains, with the catalytic site located in domain III (residues 76–381 and 416–430), which is a (β/α)₈ (TIM) barrel (Figure 1). A more detailed analysis of the active site reveals that it is virtually identical to that of imiglucerase (Figure 2), with the distances between the carboxylic oxygens of the catalytic residues, E340 and E235 (5.2 Å in molecule A and 5.1 Å in molecule B), similar to those obtained previously (Brumshtein et al. 2006) and in agreement with the distances proposed for acid–base hydrolysis (Davies and Henriessat 1995). Moreover, the three loops (loop 1, residues 345–350; loop 2, residues 393–399; and loop 3, residues 312–319) observed in previous structures (reviewed in Kacher et al. (2008)) are also seen in velaglucerase alfa. Similarly to prGCD (Shaaltiel et al. 2007), loops 2 and 3 show differences in their backbone angles and side chain orientations in the two molecules of the asymmetric unit, whereas loop 1, since it makes crystal contacts, exhibits less pronounced conformational changes (Figure 2). In the case of loop 3, a helical conformation is seen in molecule B, whereas a coiled conformation is seen in molecule A (Figure 3), as previously reported for imiglucerase (Brumshtein et al. 2006). Although the crystal was cryo-protected with 25% ethylene glycol, we did not detect any ethylene glycol molecules in the electron density map.

Imiglucerase and prGCD both contain an Arg to His mutation at residue 495, with H495 making an H-bond (2.6 Å) with the peptide carbonyl of F31. In contrast, velaglucerase alfa contains a sequence identical to that of the natural human enzyme,

Table II. RMS deviations of velaglucerase alfa compared to imiglucerase and prGCD. RMS deviations (Å) are shown for each of the two copies of the molecules in the asymmetric unit and were calculated using PyMol (www.pymol.org). The PDB codes for imiglucerase and pr-GlcCerase are 2J25 and 2V3F, respectively

	Imiglucerase-A	Imiglucerase-B	prGCD-A	prGCD-B
Velaglucerase alfa-A	0.39	0.35	0.36	0.40
Velaglucerase alfa-B	0.38	0.43	0.46	0.46

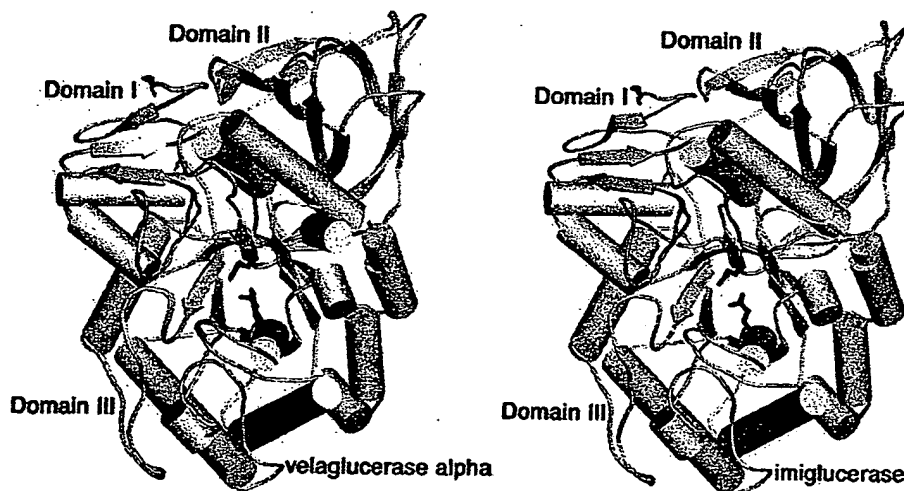


Fig. 1. Comparison of the crystal structures of velaglucerase alfa and imiglucerase. The three domains of the enzymes are colored pink (domain I, residues 1–29 and 383–414), blue (domain II, residues 30–75 and 431–497), and gray (domain III, residues 76–382 and 415–430).

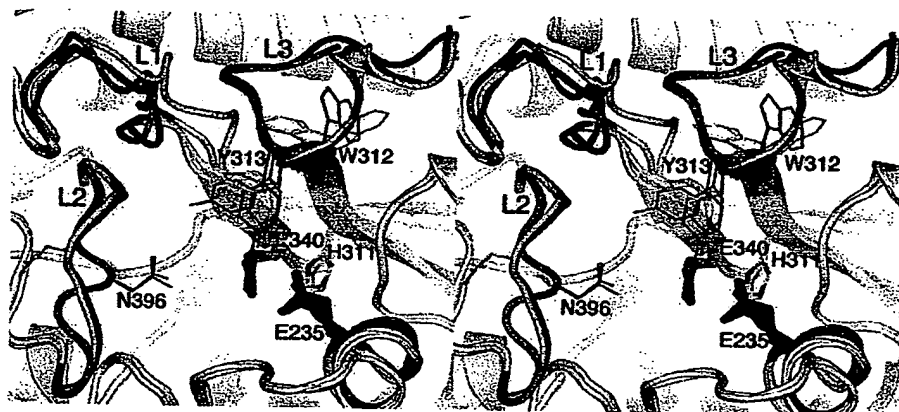


Fig. 2. Active site of velaglucerase alfa. Stereo representation of an overlay of the active sites of imiglucerase (blue and magenta) and velaglucerase alfa (yellow and green). Catalytic residues are shown as red sticks. Loops near the entrance to the active site are indicated (L1, loop 1; L2, loop 2; L3, loop 3).

with an Arg at residue 495, which does not make a similar H-bond. No major structural differences were observed in velaglucerase alfa around residue R495, relative to imiglucerase or prGCD. Two mutations which cause Gaucher disease, R496 and D474 (Figure 4) (Kawame et al. 1992; Beutler et al. 1993; Choy et al. 1998), are in close proximity to R495 near the N-terminus of GlcCerase. D474 is at the end of a β -strand, and R496 is part of a coil with no clear secondary structure, and their side-chains form a salt bridge and hydrogen bonds with each other; mutations in either of these two residues would disrupt these interactions. By analyzing the geometry and the interactions between the side chains of these two residues, and the secondary structure of the region, we conclude that R496 or D474 may be involved in stabilizing the conformation of the N-terminus of the enzyme by their side chain interactions, with disruption of these bonds resulting in a flexible N-terminus and hence in a less stable structure. However, neither of these residues interacts with R495.

Kinetic analysis

To further compare velaglucerase alfa and imiglucerase, and to determine if the mutation at residue 495 has any effect, kinetic parameters and specific activity were determined using a natural glucosylceramide (GlcCer) substrate, rather than a surrogate substrate typically used to assess enzyme activity. Velaglucerase alfa has a k_{cat} of 2100 min^{-1} , a K_m of $19 \mu\text{M}$, and a V_{max} of $0.61 \mu\text{M min}^{-1}$. Imiglucerase has a k_{cat} of 1900 min^{-1} , a K_m of $15 \mu\text{M}$, and a V_{max} of $0.56 \mu\text{M min}^{-1}$ (Figure 5). Similar K_m values were reported in the literature; GlcCerase derived from brain tissue and fibroblasts both have a K_m of $32 \mu\text{M}$ using GlcCer from Gaucher spleen (Vaccaro et al. 1982), while imiglucerase and prGCD have a K_m of 15.2 and $20.7 \mu\text{M}$, respectively, using a fluorescent GlcCer analog, C6-NBD-GlcCer (Shaaltiel et al. 2007). In addition, at a $210 \mu\text{M}$ GlcCer substrate concentration, velaglucerase alfa and imiglucerase have similar specific activities of 26 and 24 U/mg , respectively. These results

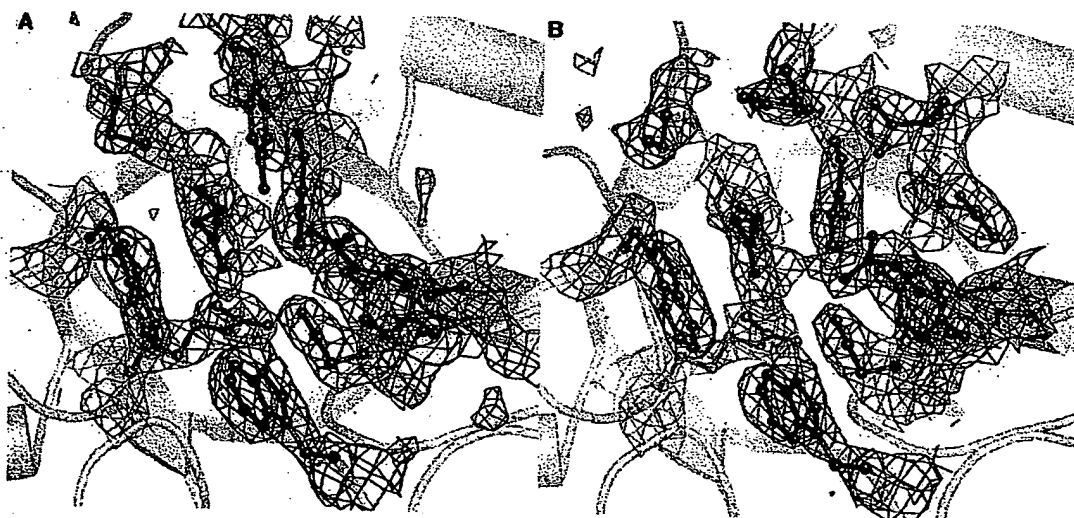


Fig. 3. Electron density around the catalytic center. Catalytic residues E235 and E340 are shown as red balls and sticks and surrounding residues are in dark gray. Contours of the $2F_o - F_c$ map are shown as a blue mesh (at 1.2σ); contours of the $F_o - F_c$ map are shown in green mesh (at 3σ) and in magenta (at -3σ). Several $F_o - F_c$ peaks are visible in the active site, but they did not overlap with the $2F_o - F_c$ map, nor are they continuous; hence, at this resolution they appear to be noise. A and B show the catalytic centers of molecules A and B, respectively, in the asymmetric unit.

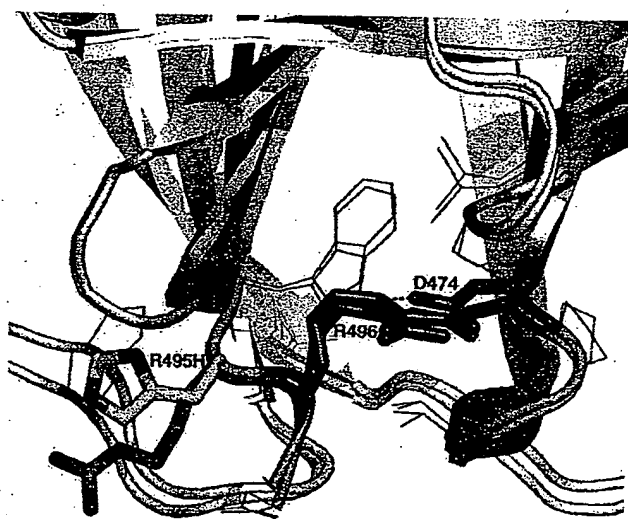


Fig. 4. Mutations at the C-terminus of GlcCerase. Imiglucerase and pr-GlcCerase contain a His at residue 495 (yellow), whereas velaglucerase alfa contains Arg (green). Mutations R496 and D474, which cause Gaucher disease, are shown in magenta. Residues within 4 Å distance of R495 and R496 are shown in cyan.

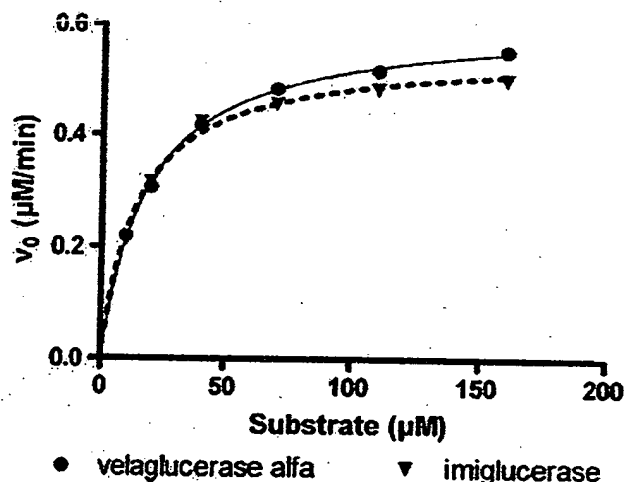


Fig. 5. Kinetic analysis of velaglucerase alfa and imiglucerase. V_{max} and K_m values were determined using a natural GlcCer substrate ($n = 2$).

demonstrate that human and CHO-cell derived GlcCerase, prepared by two different manufacturing processes, have similar enzymatic activities for the natural substrate.

Glycan composition

We next examined which sugars could be detected in the crystal structure of velaglucerase alfa. Even after partial deglycosylation using *N*-glycosidase F, two sugar residues were observed attached to residue N19 in both molecules A and B (Figure 6). One sugar was detected on N146 in molecule A whereas no sugars were detected on N146 in molecule B (Figure 6). As reported previously for imiglucerase, no sugars were detected attached to either N270 or N59 in velaglucerase alfa. It should

be noted that sugars attached to N270 have not been seen in any of the crystal structures solved to date, and sugars have been seen only occasionally on N59 (Brumshtein et al. 2006). The inability to detect sugars on either N59 or N270 is most likely due to the high flexibility of the corresponding glycan chains since nano-liquid chromatography electrospray ionization tandem mass spectrometry (nano-LC-ESI-MS/MS) analysis of intact imiglucerase (Kacher et al. 2008), and of velaglucerase alfa (see below) showed that glycan chains were attached to both these residues.

Velaglucerase alfa and imiglucerase bear distinctly different glycan chains due to the differences in their manufacture. In our comparative study of the carbohydrate content of unmodified velaglucerase alfa and imiglucerase by LC-ESI-MS, four of the five potential glycosylation sites, namely, N19, N59, N146, and N270, were observed to be fully occupied in both. As expected from the crystal structures, N462 is fully unoccupied in both, due to its buried location.

According to LC-ESI-MS analysis of glycopeptide maps, velaglucerase alfa contains primarily high-mannose type glycans, consisting of six to nine mannose units. Listed as the predominant structure in Table III, the most abundant ion present in the averaged spectra for each site corresponds to a glycan with nine mannose units. Glycan microheterogeneity was observed at each site and the less abundant structures are listed as other glycans. These other glycans consist of mannose residues with phosphorylation at the C-6 position to create a mannose-6-phosphate (M6P) residue. The lowest levels of M6P were at N19; N59 and N146 had similar but higher levels relative to N19, while N270 had the highest amount of M6P. Despite the site-specific variation in relative levels of M6P, nonphosphorylated glycans remained the predominant species for all four sites. Also observed on N59, N146 and N270 were mono-sialylated mono-antennary hybrid and complex-type structures with core fucosylation, which were quantified by glycan map analysis. These structures are consistent with a low percentage of glycosylation sites escaping kifunensine inhibition, resulting in glycan maturation and core fucosylation. In the case of hybrid-type glycans, only a single antenna matured.

The results from site-specific glycan characterization were corroborated by glycan map analysis (Figure 8), which demonstrates high-mannose type glycans consisting of six to nine mannose units with a predominant nine-mannose structure. Estimates from glycan map analysis show that the mono-sialylated mono-antennary hybrid structures account for ~2% of the total glycan pool. The map also demonstrates the presence of high-mannose glycans containing one GlcNAc-capped M6P, a result of incomplete glycan processing, as well as high-mannose glycans bearing a single M6P. Also consistent with these results were data obtained from monosaccharide compositional analysis that demonstrates approximately 0.8 mole of M6P per mole of velaglucerase alfa, and approximately 0.6 moles of M6P per mole of imiglucerase.

Site-specific glycan analysis demonstrated that imiglucerase contains primarily complex-type glycans with core fucosylation that terminate with the chitobiose tri-mannosyl core (Table IV), with an exception at the N19 site, which was observed to be devoid of fucose. These structures are as expected for GlcCerase with exoglycosidase treatment to expose the core mannose residues. Imiglucerase also contains glycan microheterogeneity at each site of glycosylation, with lower

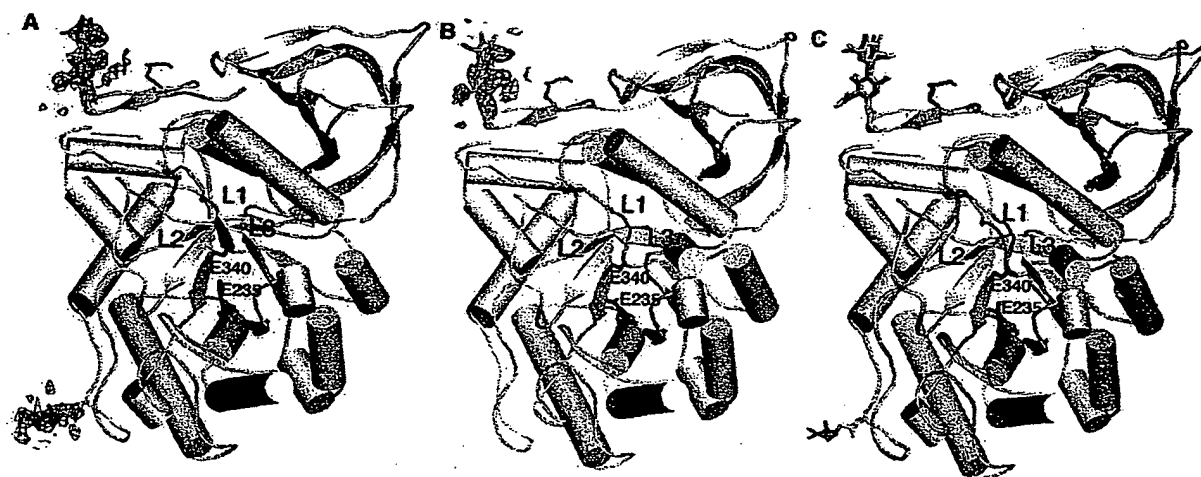


Fig. 6. Glycosylation sites seen in the crystal structure of velaglucerase alfa. $2F_o - F_c$ electron density maps are shown, which are contoured at 1.2σ in the vicinity of two of the putative glycosylation sites, N19 and N146 for molecule A, and N19 for molecule B. (A) Glycosylation sites detected in molecule A are shown in green. (B) Glycosylation site detected in molecule B is shown in yellow. (C) Superposition of the two individual molecules in the asymmetric unit reveals their similarity. In all three representations, catalytic residues E235 and E340 are shown as red sticks.

Table III. Carbohydrate composition of velaglucerase alfa. Predominant structures are those observed to be most abundant at each *N*-linked glycosylation site. The other glycans consisted mostly of high-mannose type structures (some with M6P) and with the hybrid and complex types observed at low levels ($\sim 2\%$ of the total as determined by glycan map analysis)

Glycosylation site	Predominant glycan	Other glycans
Asn19	High mannose (Man) ₉ (GlcNAc) ₂	High mannose (Man) ₆₋₈ (GlcNAc) ₂ Phosphorylated high mannose (Phos) ₁ (Man) ₈₋₉ (GlcNAc) ₂ GlcNAc-capped phosphate (Phos) ₁ (Man) ₈₋₉ (GlcNAc) ₃ Hybrid (Hex) ₂ (Man) ₃ (GlcNAc) ₃ (Fuc) ₁
Asn59	High mannose (Man) ₉ (GlcNAc) ₂	High mannose (Man) ₅₋₈ (GlcNAc) ₂ Phosphorylated high mannose (Phos) ₁ (Man) ₇₋₉ (GlcNAc) ₂ GlcNAc-capped phosphate (Phos) ₁ (Man) ₈₋₉ (GlcNAc) ₃ Hybrid (NeuAc) ₁ (Gal) ₁ (Man) ₅ (GlcNAc) ₃ (Fuc) ₁ Complex (NeuAc) ₀₋₂ (Gal) ₂ (Man) ₃ (GlcNAc) ₄ (Fuc) ₁ (Gal) ₃ (Man) ₃ (GlcNAc) ₅ (Fuc) ₁
Asn146	High mannose (Man) ₉ (GlcNAc) ₂	High mannose (Man) ₆₋₈ (GlcNAc) ₂ Phosphorylated high mannose (Phos) ₁ (Man) ₇₋₉ (GlcNAc) ₂ GlcNAc-capped phosphate (Phos) ₁ (Man) ₉ (GlcNAc) ₃ Hybrid (NeuAc) ₁ (Gal) ₁ (Man) ₅ (GlcNAc) ₃ (Fuc) ₁
Asn270	High mannose (Man) ₉ (GlcNAc) ₂	High mannose (Man) ₆₋₈ (GlcNAc) ₂ Phosphorylated high mannose (Phos) ₁ (Man) ₈₋₉ (GlcNAc) ₂ GlcNAc-capped phosphate (Phos) ₁ (Man) ₉ (GlcNAc) ₃ Hybrid (Gal) ₁ (Man) ₇ (GlcNAc) ₃ (Fuc) ₁ (NeuAc) ₁ (Gal) ₁ (Man) ₅ (GlcNAc) ₃ (Fuc) ₁
Asn462	Not detected	Complex (NeuAc) ₂ (Gal) ₂ (Man) ₃ (GlcNAc) ₄ (Fuc) ₁ Not detected

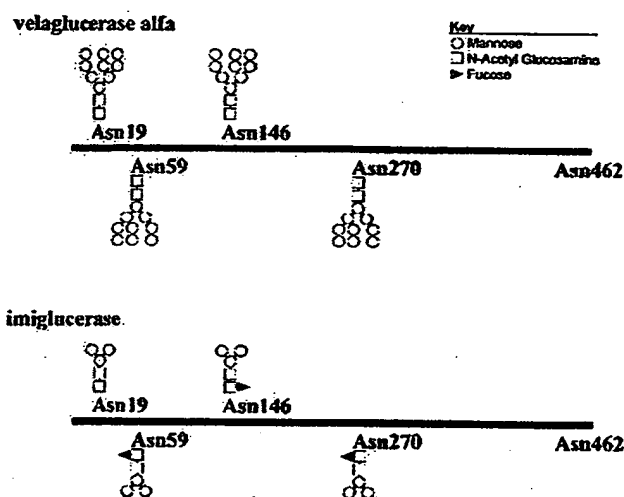


Fig. 7. Glycan structures of velaglucoerose alfa and imiglucerase. Predominant *N*-linked carbohydrate structures on velaglucoerose alfa (top) and imiglucerase (bottom) are shown graphically at their relative positions along the protein backbone.

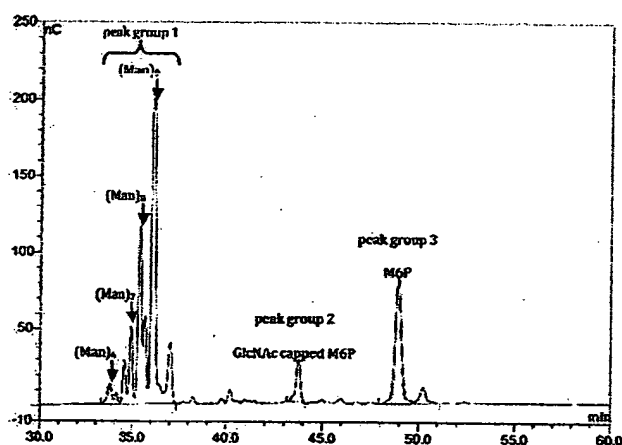


Fig. 8. Glycan map analysis of velaglucoerose alfa. Glycans released by *N*-glycosidase F were analyzed by anion-exchange chromatography with amperometric detection. The method resolves glycans based on negative charge where peak group 1 corresponds to high-mannose type neutral glycans that are resolved into multiple peaks according to the number of mannose units, peak group 2 corresponds to high-mannose type glycans with one M6P that retained its GlcNAc cap (one negative charge), and peak group 3 corresponds to high-mannose type glycans containing one fully processed M6P (two negative charges). In peak group 1, smaller peaks are resolved that correspond to positional isomers of the various oligomannose types observed.

levels of core structures terminating with *N*-acetylglucosamine (GlcNAc) that are likely a result of incomplete digestion with *N*-acetylglucosaminidase. At N146 and N270, high-mannose type glycans were observed containing five to six mannose units with one M6P.

The glycan graphics shown in Figure 7 help to visualize the predominant structures for both forms of GlcCer as described in Tables III and IV. These structures were consistent with glycan types and levels observed with glycan map analysis as well as with previous reports (Van Patten et al. 2007). In the current

Table IV. Carbohydrate composition of imiglucerase. Predominant structures are those observed to be most abundant at each *N*-linked glycosylation site. The other glycans consisted mostly of core structures with additional GlcNAc and high-mannose structures with M6P

Glycosylation site	Predominant glycan	Other glycans
Asn19	Complex (Man) ₃ (GlcNAc) ₂	Complex (Man) ₃ (GlcNAc) ₃
Asn59	Complex (Man) ₃ (GlcNAc) ₂ (Fuc) ₁	Complex (Man) ₃ (GlcNAc) ₃ (Fuc) ₁
Asn146	Complex (Man) ₃ (GlcNAc) ₂ (Fuc) ₁	Complex (Man) ₃ (GlcNAc) ₃₋₄ (Fuc) ₁ Phosphorylated high mannose (Phos) ₁ (Man) ₅₋₆ (GlcNAc) ₂ GlcNAc-capped phosphate (Phos) ₁ (Man) ₅₋₆ (GlcNAc) ₃
Asn270	Complex (Man) ₃ (GlcNAc) ₂ (Fuc) ₁	Complex (Man) ₃ (GlcNAc) ₃₋₄ (Fuc) ₁ Phosphorylated high-mannose (Phos) ₁ (Man) ₅₋₆ (GlcNAc) ₂ GlcNAc-capped phosphate (Phos) ₁ (Man) ₅₋₆ (GlcNAc) ₃

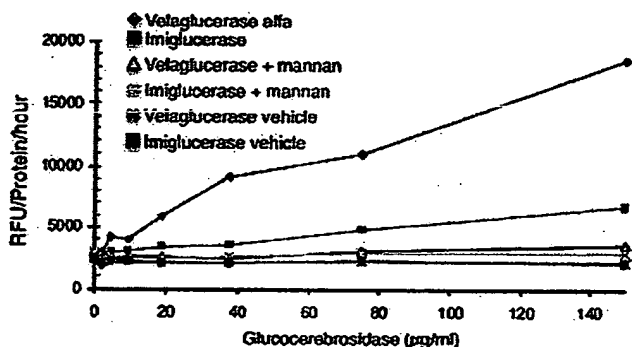


Fig. 9. Velaglucoerose alfa and imiglucerase internalization into differentiated macrophages. The ordinate of the graph represents the fluorescence data normalized for the cellular protein concentration and incubation time (RFU/ μ g/h). The GlcCer dose is shown on the abscissa.

study, the glycans of prGCD were not characterized, but earlier studies demonstrated the presence of core α -(1,2)-xylose and core α -(1,3)-fucose (Shaaltiel et al. 2007), which are unique to plant-derived proteins and would not be expected to be present on either velaglucoerose alfa or imiglucerase.

Internalization by macrophages

Internalization of proteins by endocytosis is highly dependent upon their carbohydrate composition and has been well characterized (Kornfeld 1986). A comparison of the internalization rate of velaglucoerose alfa to that of imiglucerase in U937-derived macrophages demonstrated that velaglucoerose alfa is internalized approximately 2.5-fold more efficiently than imiglucerase (Figure 9). Internalization of both enzymes could be inhibited by the addition of mannan to the culture medium, demonstrating that internalization was mediated via mannose receptors; moreover, U937 cells were shown by immunohistochemistry to express mannose receptors (CD206) (data not shown). It should be noted that during optimization of this assay, variations in results were obtained when different culture media were used.

Therefore, additional research will be required to determine the exact nature of the uptake since different mannose receptors exist, which may be involved in this cellular internalization. In contrast, the addition of M6P to the culture medium had no effect, confirming that the M6P receptor is not involved in internalization (data not shown). Since velaglycerase alfa and imiglycerase display similar kinetic parameters, specific activities, and structural features, the different rates of internalization can be ascribed to differences in glycosylation patterns between velaglycerase alfa and imiglycerase, with the increased rate of internalization of velaglycerase alfa likely due to the expression of longer chain high-mannose type glycans compared to the core mannose structures found on imiglycerase.

Conclusions

In summary, the X-ray structure of velaglycerase alfa is very similar to those of recombinant GlcCerases produced in other expression systems, with the R495H mutations found in imiglycerase and prGCD having no effect on the secondary structure. The main difference between imiglycerase and velaglycerase alfa concerns their glycan structures, with the latter containing longer chain high-mannose type glycans compared to the core mannose structures found on imiglycerase. This difference in glycosylation appears to lead to the increased cellular uptake of velaglycerase alfa over imiglycerase. The role of protein glycosylation in cellular uptake is widely established in many cell types (Barton et al. 1991). However, while the function of the macrophage mannose receptor (MR; CD206) in internalization of mannoseylated proteins is well characterized (East and Isacke 2002), a growing family of carbohydrate-binding receptors have been implicated in diverse macrophage functions including removal and disposal of endotoxin (Ono et al. 2006), utilization of secreted lysosomal enzymes (Abe et al. 2008), phagocytosis (Kang et al. 2005), and regulation of the innate immune response to microbial pathogen-associated structures (Garner et al. 1994). Thus, the differences in uptake observed between imiglycerase and velaglycerase-alfa can be attributed to differences in affinity for CD206, or alternatively could be due to differential uptake mediated by other macrophage mannose receptors such as Endo180. This observed increase in cellular uptake of velaglycerase-alfa over imiglycerase can be envisioned to lead to a more rapid time to improvement of clinical parameters and potentially increased therapeutic efficacy.

Material and methods

Crystallization, structure determination, and refinement

Velaglycerase alfa was partially deglycosylated (Kacher et al. 2008) prior to crystallization, as previously described for imiglycerase (Dvir et al. 2003; Premkumar et al. 2005), using *N*-glycosidase F (88 h at 25°C), which removes carbohydrate chains from proteins and peptides by cleaving the amide bonds between Asn residues and *N*-acetylglucosamine (GlcNAc) (Han and Martinage 1992), but does not necessarily remove all carbohydrate chains from native proteins. Subsequent to *N*-glycosidase F-treatment, velaglycerase alfa was diluted in the crystallization buffer (10 mM citrate pH 5.5, 7% (v/v) ethanol, 0.02% (w/v) Na₂S₂O₃) and passed through a Centricon YM-30 centrifugal filter device with a molecular mass cut-off of ~30 kDa, to give a final concentration of 4–5 mg/mL. Ve-

laglycerase alfa crystals were obtained by micro-batch crystallization under oil (Chayen et al. 1990) using a Douglas Instruments Oryx6 robot. The crystallization solution had a 1:1 ratio of the concentrated enzyme solution and of 1 M (NH₄)₂SO₄/0.1 M HEPES, pH 7.0, containing 0.5% (w/v) PEG8000. Crystallization was performed under Al's oil (D'Arcy et al. 1996) (1:1 ratio of paraffin and silicone liquid oils) for 5–14 days at 20°C. Data were collected on beam line ID14eh2 at the ESRF synchrotron (Grenoble, France). Crystals were cryo-protected with a 25% ethylene glycol solution, mounted, and flash cooled to 100 K. X-ray diffraction images were processed using HKL2000 and scaled with SCALEPACK (Otwinowski et al. 1997). The structure was solved using the molecular replacement method based on PDB 2J25 (Brumshtein et al. 2006) and refined with Refmac5 (Murshudov et al. 1997). During the course of refinement, the electron density map showed significant improvement, and putative sugars could be seen adjacent to N19 and N146 for molecule A, and adjacent to N19 for molecule B. Table I summarizes data collection and processing. Structures and structure factors were deposited in the PDB (code 2WKJ).

Enzyme kinetics and specific activity

The novel enzymatic activity assay described below measures the ability of GlcCerase to release glucose from GlcCer obtained from Gaucher spleen (Matreya LLC, PA, Cat. no. 1057). Velaglycerase alfa (drug substance lot EP06-003) and imiglycerase (commercial product lot C7036C01) were assayed. The released glucose was quantified by anion-exchange chromatography equipped with a pulsed amperometric detector. The appropriate amount of GlcCer in chloroform/methanol (2:1, v/v) was dried by a SpeedVac in the presence of 0.2 M taurocholic acid in methanol and 20% (v/v) oleic acid in chloroform/methanol (2:1). The dried pellet was reconstituted in the 0.1 M citrate/0.2 M phosphate buffer (pH 5.0) and diluted to the desired concentrations. Enzyme samples were diluted to a concentration of 0.2 ng/μL with the dilution buffer (50 mM sodium citrate, pH 6.0 with 0.75 mg/mL BSA) and 2 ng of enzyme was incubated for 30 min at 37°C with serial dilutions of GlcCer in a 110 μL reaction volume. The reaction was stopped by heat denaturing samples at 100°C for 5 min. Sample manipulations were internally controlled by adding 100 μL of a galactosamine (GalN) solution to the reaction mixture. Dionex OnGuard II RP cartridges were used to remove the detergent and lipid. The analysis was carried out on a Dionex high-performance anion-exchange chromatography device, coupled with a pulsed amperometric detection apparatus (HPAE-PAD), using a CarboPac PA-10 analytical column equipped with a CarboPac PA-10 guard column. An isocratic flow of 6 mM NaOH at 0.25 mL/min for 25 min was used to separate monosaccharides (Glc and GalN). The amount of glucose (Glc) was calculated from linear regression analysis of GalN and Glc standards in the range of 10–480 pmol per injection. The assay was carried out in a range of substrate concentrations of 5–150 μM, and obeyed Michaelis-Menten kinetics, thus permitting assignment of *K_m* and *V_{max}* values.

Site-specific characterization of glycans

Velaglycerase alfa (drug substance lot EP06-003, Shire Human Genetic Therapies, Hampshire, UK) and imiglycerase (commercial product lot HA163BL) were prepared for enzymatic digestion by reductive denaturation with DTT, followed by and

cysteine alkylation with iodoacetic acid. Alkylated samples were digested first with the endoproteinase Lys-C (Roche Diagnostics GmbH, Mannheim, Germany) (1:42 enzyme to substrate ratio, w/w, for 6 h at 37°C), followed by digestion with endoproteinase Glu-C (1:25 enzyme to substrate ratio, w/w, for 16 h at room temperature). Digested samples were analyzed by peptide mass mapping using reversed phase chromatography with in-line UV (214 nm) and electrospray ionization with mass spectrometric detection (LC-ESI-MS). By comparing the peptide maps before and after glycan release using *N*-glycosidase F (New England Biolabs, Ipswich, MA), the five potential glycosylation sites were identified. The glycan mass was calculated by subtracting the expected peptide mass from the observed glycopeptide masses. Using software to match the observed glycan masses with potential monosaccharide compositions, glycan compositions for each site were determined. To verify monosaccharide compositions, treatments (according to manufacturer's recommendations) with neuraminidase (Roche Diagnostics GmbH), alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany), and α -mannosidase (Glyko, Inc., Hayward, CA) were used to verify the presence of sialic acid, phosphate, and alpha-linked mannose, respectively. MS/MS fragmentation analysis was used to verify glycan phosphorylation.

Glycan map analysis

The procedure involves heat denaturation of the protein at 100°C for 3–4 min in the presence of 0.5% SDS, followed by enzymatic release of glycans with *N*-glycosidase F (Prozyme, San Leandro, CA). Velaglycerase alfa (drug substance lot EP06-001, Shire Human Genetic Therapies) was incubated with *N*-glycosidase F (30 mU/3 μ L) for 4–6 h at 37°C with 0.9% NP40, followed by a second addition of *N*-glycosidase F, and an additional 17–19 h incubation at 37°C. Analysis of the released glycans was performed by HPAE-PAD, using a CarboPac PA-1 analytical column equipped with a CarboPac PA-1 guard column (Dionex, Sunnyvale, CA). Glycans were applied to the column in 12 mM sodium acetate/100 mM NaOH, followed by elution with a 12–300 mM sodium acetate gradient (6.4 mM/min) in 100 mM NaOH in 45 min. Using a flow rate of 1 mL/min and the column at ambient room temperature, glycans elute in the order of increasing negative charge.

Cellular internalization

Human U937 cells were cultured in growth media containing RPMI 1640 with 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, and 10% FBS. Treatment with phorbol myristate acetate (PMA) for 3 days was used to induce differentiation into macrophages (Amento et al. 1984). The U937-derived macrophages were seeded into 96-well microtiter plates at 50,000 cells per well in growth medium, and allowed to adhere to the plates for 48 h. Seeded macrophages were incubated for 3 h with equimolar preparations of velaglycerase alfa (drug substance lot FEC06-003, Shire Human Genetic Therapies) or imiglycerase (Cerezyme®; commercial product lot C7036C01, Genzyme, Cambridge, MA) at pH 7.5, in growth medium containing RPMI 1640 devoid of phosphate, 0.1% BSA, 10 mM HEPES, pH 7.5, 2 mM L-glutamine, 1 mM DTT, and 10 mM CaCl₂. In all assays, the cells were treated with GlcCerase for a 3-h duration which was previously determined to be in the

linear range of internalization. For dose response curves utilized to demonstrate mannose-receptor specificity, 10 mg/mL mannan was used to antagonize the receptor. After a series of wash steps (wash buffer: 0.05 M Tris, 0.138 M NaCl, 0.0027 M KCl, with 0.05% Tween 20, 0.5% BSA, pH 8.0), the cells were lysed (lysis buffer: 10 mM Tris pH 8.0, 0.5% NP40, 0.2% deoxycholate, Complete Mini Protease Inhibitor Cocktail Tablets in EASYpacks and PhosSTOP Phosphatase Inhibitor Cocktail Tablets in EASYpacks, Roche Applied Science), and the internalized GlcCerase was quantified by an assay employing the synthetic substrate, 4-methylumbelliferyl- β -D-glucopyranoside (4-MU-glc), which releases a fluorescent product upon cleavage. The protein content in the well was determined (BCA method according to the manufacturer's protocol) and was used to normalize the assay signal to total protein from each sample. The assay signal for the GlcCerase samples was tested in vitro to determine the extent of activity or signal disparity between the two drugs, and there was no difference in activity (data not shown). For these assays, 2-fold serial dilutions of velaglycerase alfa and imiglycerase (starting at 30 nM enzyme) were made in the assay lysis buffer and tested using the 4-MU-glc enzymatic activity assay. Plates were read with a Perkin Elmer Envision multi-label plate reader.

Funding

Shire Human Genetic Therapies, Inc.

Acknowledgements

J.L. Sussman is the Morton and Gladys Pickman Professor of Structural Biology, and A.H. Futerman is the Joseph Meyerhoff Professor of Biochemistry at the Weizmann Institute of Science. The contribution of Meng Wu, for technical assistance is gratefully acknowledged. We are grateful to Dr. Hilary Voet (Faculty of Agriculture, The Hebrew University, Rehovot) for invaluable discussions concerning the statistical analysis of the choice of space groups.

Conflict of interest statement

None declared.

Abbreviations

CHO, Chinese hamster ovary; ERT, enzyme replacement therapy; GA-GCB, velaglycerase alfa; GlcCer, glucosylceramide; GlcCerase, acid- β -glucosidase; M6P, mannose-6-phosphate; prGCD, GlcCerase expressed in transgenic carrot cells; RMSD, root mean square deviation.

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In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment G

Letter from FDA to Transkaryotic Therapies, Inc., dated January 12, 2004,
providing the IND number and showing the date of receipt by FDA of the IND



DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Rockville, MD 20857

JAN 19 2004

IND 61,220

Transkaryotic Therapies, Inc.
Attn: Suzanne L. Bruhn, Ph.D.
Vice President, Regulatory Affairs
700 Main Street
Cambridge, MA 02139

Dear Dr. Bruhn:

We acknowledge receipt of your Investigational New Drug Application (IND) submitted under section 505(i) of the Federal Food, Drug, and Cosmetic Act. Please note the following identifying data:

IND Number Assigned:	61,220
Sponsor:	Transkaryotic Therapies, Inc.
Name of Drug:	Gene-Activated [®] Glucocerebrosidase (GA-GCB, DRX008A)
Date of Submission:	December 30, 2003
Date of Receipt:	December 31, 2003

Studies in humans may not be initiated until 30 days after the date of receipt shown above. If, on or before January 30, 2004, we identify deficiencies in the IND that require correction before human studies begin or that require restriction of human studies, we will notify you immediately that (1) clinical studies may not be initiated under this IND ("clinical hold") or that (2) certain restrictions apply to clinical studies under this IND ("partial clinical hold"). In the event of such notification, you must not initiate or you must restrict such studies until you have submitted information to correct the deficiencies, and we have notified you that the information you submitted is satisfactory.

It has not been our policy to object to a sponsor, upon receipt of this acknowledgement letter, either obtaining supplies of the investigational drug or shipping it to investigators listed in the IND. However, if the drug is shipped to investigators, they should be reminded that studies may not begin under the IND until 30 days after the IND receipt date or later if the IND is placed on clinical hold.

As sponsor of this IND, you are responsible for compliance with the Federal Food, Drug, and Cosmetic Act and the implementing regulations (Title 21 of the Code of Federal Regulations). Those responsibilities include (1) reporting any unexpected fatal or life-threatening adverse experience associated with use of the drug by telephone or fax no later than 7 calendar days after initial receipt of the information [21 CFR 312.32(c)(2)]; (2) reporting any adverse experience associated with use of the drug that is both serious and unexpected in writing no later than 15 calendar days after initial receipt of the information [21 CFR 312.32(c)(1)]; and (3) submitting annual progress reports [21 CFR 312.33].

Please forward all future communications concerning this IND in triplicate, identified by the above IND number, to the following address:

U.S. Postal Service/Courier/Overnight Mail:
Food and Drug Administration
Center for Drug Evaluation and Research
Division of Metabolic and Endocrine Drug Products, HFD-510
Attention: Fishers Document Room, 8B-45
5600 Fishers Lane
Rockville, Maryland 20857

If you have any questions, call me at (301) 827-6416.

Sincerely,

{See appended electronic signature page}

Patricia Madara
Regulatory Project Manager
Division of Metabolic & Endocrine Drug Products
Office of Drug Evaluation II
Center for Drug Evaluation and Research

**This is a representation of an electronic record that was signed electronically and
this page is the manifestation of the electronic signature.**

/s/

Patricia Madara
1/12/04 02:28:37 PM

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNANOSE PROTEINS AND METHODS OF MAKING HIGH
MANNANOSE PROTEINS

Attachment G1

A written record of the discussion that occurred on January 28, 2004 regarding
modification of the protocol

**FDA CONTACT REPORT
DRX008A**

Contact:	Pat Madara, Metabolic and Endocrinologic Group
	Phone: 301-827-6416
Date:	28 Jan 2004
Time:	10:30

TKT Participants: Steve Schmitz (SS)

Executive Summary:

- Pat Madara telephoned to say that Dr. Pariser, the medical reviewer for the IND submission, informed her that she had not received the protocol amendment, which incorporated the changes discussed at the Pre-IND meeting.
- If she did not receive the amendment, the program would be placed on clinical hold.
- I informed her that we were prepared to send out the amendment within the next 1-2 days.

Action Item:

- Send out the protocol amendment to Pat Madara.

Signature:



Date:

28 Jan 2004

Copy List: W. Aliski
 R. Fram

RA Archives (original)
RA Chronology

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment G2

Letter from Transkaryotic Therapies, Inc. to FDA dated March 11, 2004,
concerning amendment of protocol

11 March 2004

Patricia Madara
Regulatory Project Manager
Division of Metabolic and Endocrine Drug Products
Office of Drug Evaluation II
Center for Drug Evaluation and Research
5600 Fishers Lane
Rockville, MD 20857

RE: IND# 61,220
Amendment 2.0, Clinical Protocol TKT025
Serial No. 002

Product Name: Mannose-terminated, Gene-Activated[®] Glucocerebrosidase,
(GA-GCB, DRX008A)

Dear Ms. Madara:

Please find enclosed a copy of Amendment 2.0 to Clinical Protocol TKT025 (Attachment 1), and a document entitled, "Listing of Changes in Amendment No. 2 to TKT Clinical Protocol No. TKT025 (Attachment 2).

The changes include:

- 1) Addition of a MRI of the lumbar spine. Originally, the MRI evaluation examined only the femora and abdomen. However, in order to enhance the assessment of bone marrow involvement by the Bone Marrow Burden Score (see below), an MRI of the lumbar spine is required.
- 2) The addition of an exploratory clinical activity variable, the bone marrow burden score. This score is obtained by evaluating MRI images of both the axial (lumbar spine) and peripheral (femora).
- 3) Clarification of timing of vital sign determination
- 4) Itemization, by "bulleting", in the Schedule of Events, to specify electrocardiogram testing at Weeks 21 and 33.
- 5) Updating of the Informed Consent to describe the additional tests mentioned above. In addition, a statement regarding the potential eligibility of a patient who declined to enter the study, to receive approved therapy (i.e., imiglucerase), was deleted. TKT believed that the statement, as written in the Amendment 1.0, had potential to be misleading to patients. We were concerned that a patient could possibly interpret the previous wording to mean that, in the event that he declined to participate in the study, the Sponsor would provide imiglucerase, a currently approved therapy for Gaucher disease.

If you have any questions or comments, please telephone me at 617-613-4364. Thank you for your consideration.

Sincerely,

A handwritten signature in black ink, appearing to read "Stephen M. Schmitz". The signature is fluid and cursive, with the first name "Stephen" being more prominent than the last name "Schmitz".

Stephen M. Schmitz, M.D., M.P.H.
Director, Safety and Regulatory Affairs
Transkaryotic Therapies, Inc.

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment G3

FDA communication to Transkaryotic Therapies, Inc. dated May 20, 2004,
concerning amendment to protocol

AGENCY CORRESPONDENCE

GCB

Contact(s)	Pat Madara, Project Manager Div. of Metabolic and Endocrinologic Drug Products/CDER/FDA 301-827-6416
Date	20 May 2004
Time	1400 and 1415 (Hours and Minutes in Military Time)

Agency Participants

Pat Madara, Project Manager (PM)

Re: IND 61,220 – DRX008A (glucocerebrosidase, GA-GCB)

TKT Participants

Suzanne L. Bruhn, VP, Reg. Affairs (SB)

Alyssa Sonntag, Project Manager, Reg. Affairs (AS)

Executive Summary

- FDA has no comments on the blinding procedure to be implemented for the analysis of liver and spleen volumes from MRI scans taken for Study TKT025. TKT will make no changes to the current version of the blinding procedure (dated 7 April 2004).

Summary

At 1400, SB called PM to inquire on the status of FDA's review of the blinding procedure for analysis of liver and spleen volumes from the MRI scans taken for Study TKT025, which was submitted to the IND in Serial 003 on 7 April 2004. PM stated that the Medical Officer's review of this submission had been completed but she would need to confirm if there were any comments to relay to the sponsor from that review. PM stated that she would contact us soon with this information, but we should call her again after 2 weeks if we had not heard from her. PM will be out of the office during the last week of May and the first week of June.

PM called back at 1415 and informed SB that she had pulled the Medical Officer's review and that it was "safe to proceed [with the blinding procedure] as amended." Therefore, TKT will make no changes to the current version of the blinding procedure (dated 7 April 2004).

Serial Submissions Discussed

Serial 003, submitted to IND 61,220 on 7 April 2004

Action Item(s)

- None

Signature Alyssa Jountz **Date** 24 May 2004

Copy List Regulatory Chronology (Original w/ signature)

S. Bruhn

R. Fram

S. Zildjian

N. Wyant

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

**Assignee: Shire Human Genetic Therapies,
Inc.**

**Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS**

Attachment H

**Letter from FDA to Shire Human Genetic Therapies, Inc. indicating the date the
IND was put on clinical hold**

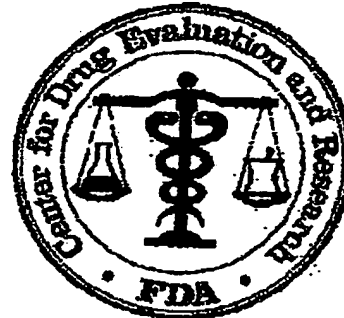
REGULATORY AFFAIRS
RECEIVED

NOV 28 2006

FAX

SHIRE HGT

**FOOD AND DRUG ADMINISTRATION
DIVISION OF GASTROENTEROLOGY PRODUCTS**
Center for Drug Evaluation and Research, HFD-180
10903 New Hampshire Ave, Silver Spring, MD 20993-0002



To: Nikhil S. Mehta, Ph.D.

From: Ryan Barraco

Fax: 617-613-4444

Fax: 301-796-9905

Phone: 617-613-4531

Phone: 301-796-0846

Pages, including cover sheet: 8

Date: November 28, 2006

Re: IND 61,220 for GA-GCB – Full Clinical Hold Letter

Comments:

Courtesy Fax

THIS DOCUMENT IS INTENDED ONLY FOR THE USE OF THE PARTY TO WHOM IT IS ADDRESSED AND MAY CONTAIN INFORMATION THAT IS PRIVILEGED, CONFIDENTIAL AND PROTECTED FROM DISCLOSURE UNDER APPLICABLE LAW. If you are not the addressee, or a person authorized to deliver the document to the addressee, you are hereby notified that any review, disclosure, dissemination or other action based on the content of the communication is not authorized. If you have received this document in error, please immediately notify us by telephone and return it to us at the above address by mail. Thank you.

**DEPARTMENT OF HEALTH & HUMAN SERVICES**

Public Health Service

Food and Drug Administration
Rockville, MD 20857

IND 61,220

FULL CLINICAL HOLD

Shire Human Genetic Therapies
Attention: Nikhil Mehta, Ph.D.
Vice President, Global Regulatory Affairs
700 Main Street
Cambridge, MA 02139

Dear Dr. Mehta:

Please refer to your Investigational New Drug Application (IND) submitted December 30, 2003, received December 31, 2003, under section 505(i) of the Federal Food, Drug, and Cosmetic Act for Gene Activated® Glucocerebrosidase (GA-GCB).

We also refer to your amendment dated August 3, 2006 (serial # 035), and to the November 20, 2006, telephone conversation between you and our Division, in which you were notified that your IND is on clinical hold and any proposed studies may not be initiated. The following are the specific deficiencies [21 CFR 312.42(b)] and the information needed to resolve these deficiencies.

Insufficient information to assess risks to human subjects [21 CFR 312.42(b)(2)(ii)].

Clinical Hold Deficiencies

The chemistry, manufacturing, and controls (CMC) amendment dated August 3, 2006, did not demonstrate that the two manufacturing processes for Gene Activated® Glucocerebrosidase (GA-GCB) yield drug substances (DS) with comparable physicochemical characteristics. The comparability data provided in this amendment revealed that there were the following physicochemical differences between DS produced by the two processes:

- a. For the glycan mapping, the total percentage of Group 1 carbohydrates is different between the two DS, and it appears that relative proportions of individual peaks within Group 1 are different in the two DS.
- b. Although the predominant peaks C, D, and E in IEX-HPLC constitute more than 75% of the total peak area and meet the acceptance criteria, there is a significant shift in the proportion of each peak, which does not appear to be due to assay variability.
- c. The pattern of bands detected by IEF gels Coomassie stained is different in DS manufactured with the serum containing process (E303-006) versus DS

IND 61,220
Page 2

manufactured with the animal-free process (EP06-003). At least one additional acidic species is present in E303-006.

As you intend to use the DS manufactured using the new manufacturing process in your proposed Phase 3 clinical study, and as this DS has not been evaluated in pre-clinical or clinical studies, insufficient information exists with this DS to assess the risks to human subjects for the proposed Phase 3 clinical investigation.

Information needed to resolve clinical hold deficiencies

1. You must demonstrate the comparability of DS by the two manufacturing processes as set forth in the "Guidance for Industry Q5E Comparability of Biotechnological/Biological Products Subject to Changes in their Manufacturing Process."
2. Alternatively, you may propose to perform your Phase 3 clinical study using the same DS administered in the completed Phase 1/2 study and in the pre-clinical testing conducted in support of this Phase 1/2 study.
3. Alternatively, you may propose to repeat pre-clinical and clinical studies with DS manufactured using the new manufacturing method, which are needed to support the proposed Phase 3 clinical study. These studies are to include:
 - a. A head-to-head comparison of the two DS in *in vitro* and *in vivo* pharmacology studies to demonstrate comparability of the two DS on the primary pharmacological effect of GA-GCB.
 - b. A head-to-head tissue distribution comparison of the two DS in Sprague Dawley rats of both sexes.
 - c. A clinical study to assess the pharmacokinetics, pharmacodynamics, and preliminary safety of GA-GCB administration.

Until you have submitted the required information, and we notify you that you may initiate the trial, you may not legally conduct the identified clinical study under this IND.

Please identify your response to the clinical hold issues as a "CLINICAL HOLD COMPLETE RESPONSE." To facilitate a response to your submission, submit this information in triplicate to the IND. In addition, send a copy of the cover letter to Ryan Barraco.

Following receipt of your complete response to these issues, we will notify you of our decision within 30 days.

In addition, we have the following recommendations and requests that are important for product development, but are not clinical hold issues at this time. Your responses to any non-hold issues should be addressed in a separate amendment to the IND.

IND 61,220
Page 3

CMC

Regarding characterization and release testing:

1. You are currently measuring enzymatic activity using a surrogate substrate. In order to properly characterize DS and drug product (DP), measurements of the K_m and k_{cat} kinetic parameters using a physiologically relevant substrate should be performed.
2. Routine DS and DP release testing should also include (i) measurements of the K_m and k_{cat} kinetic parameters using a physiologically relevant substrate, and (ii) a quantitative assessment of receptor binding and uptake by macrophages. Please note that glycan mapping is not considered to be a potency assay from a regulatory perspective.
3. An in-depth characterization of the glycan structures in GA-GCB, with information on branching and size of the glycan chains, mannose-6P content and residual content of NANA should be performed. Adequate assays that allow for control of carbohydrate content and structure should be included in release testing.
4. The area for each peak identified in groups 1, 2 and 3 of the glycan mapping assay should be specified in your release testing.
5. N-terminal sequencing and Western blotting for identity, and Ion Exchange Chromatography for purity were not performed at release testing for the clinical lots of DS. These tests assess critical product attributes and should be maintained as release tests.
6. Your current DS acceptance criteria for RP-HPLC and SE-HPLC are $\geq 94\%$ of the main peak area. From the results of batch analysis, it appears that on average, RP-HPLC purity is about 98% and SE-HPLC is about 97%. Acceptance criteria should be established based on process capability and manufacturing experience, and we recommend establishing more stringent specifications.
7. It appears that the new process generates a DS with a higher percent of aggregates (by comparison of batch analysis results). These aggregates should be characterized, and an orthogonal method to detect aggregates should be used to validate SE-HPLC.
8. It is not clear whether studies were performed to detect impurities that could arise from the DP manufacturing process. The increase in aggregation of GA-GCB during DP manufacturing should be documented, and procedures implemented to minimize aggregate formation.

IND 61,220
Page 4

9. You have significantly changed DP acceptance criteria for SE-HPLC and RP-HPLC to $\geq 92\%$ main peak area. Although you justify these acceptance criteria based on limited manufacturing experience, they appear to allow for excessive amounts of aggregates and impurities in the DP. Acceptance criteria should be established based on process capability and manufacturing experience, and we recommend establishing more stringent specifications.

Regarding the manufacturing process:

10. You provided flow charts for the manufacturing process that include in-process controls. It appears that the only in-process control in the purification step is protein recovery. We recommend inclusion of additional in-process tests that could provide information on purity such as, but not limited to, SDS-PAGE reducing and non-reducing.
11. You stated that impurities derived from the culture medium, such as plant hydrolysates, kifunensine and DTT will be removed during the purification process. You should provide supportive data for the above claims. Presence of impurities should be assessed and specified at critical steps, and in lot release. Alternatively, removal of the process-related impurities must be validated.
12. Acceptance criteria for HCP content should be modified to reflect the actual capability of the process to remove these impurities. Currently, acceptance criterion is <200 ng/mg, and the actual results range from 7 to 23 ng/mg.
13. You state that antibodies have been raised against protein lysates from cells growing in serum-free and serum-containing medium. Please clarify which antibodies have been used to develop the ELISA and Western blotting assays.

Regarding stability:

14. It is important to demonstrate that critical drug potency parameters are not altered at the indicated storage temperature. Please include the following assays in your stability testing program for DS and DP:
 - a. Macrophage uptake assay and receptor binding assay.
 - b. Measurements of the K_m and k_{cat} kinetic parameters.
 - c. All assays should be evaluated for stability indicating potential.
15. Please refer to ICH Q5C for guidance on stability studies for biotechnology products.

IND 61,220
Page 5

Regarding cell banks:

16. You provided a stability program for MCB and WCB of up to four years. Viability and growth should be assessed at later times as well, to ensure that a constant supply of starting material is reproducibly available.
17. Please submit data regarding viral clearance by filtration for review as soon as they are available.

Clinical/Statistical

Regarding your proposed Phase 3 clinical protocol:

18. Your protocol contains a large number of secondary endpoints. If you intend to use any of these secondary endpoints to support the indication for the treatment of type 1 Gaucher disease with GA-GCB, you will need to include in your statistical analysis plan a proposal for evaluating these endpoints in a statistically rigorous manner that accounts for multiplicity.
19. Please provide a rationale for performing pharmacokinetic sampling to evaluate the multiple-dose pharmacokinetics of GA-GCB at Week 37.
20. Your protocol excludes from study participation patients who are anti-imiglucerase IgG antibody positive. As stated at the End of Phase 2 (EOP2) meeting on January 11, 2006, it is likely that at least some of the patients in clinical practice who transition from Cerezyme® to GA-GCB will be IgG anti-imiglucerase antibody positive. We recommend that the inclusion criteria be broadened to include type 1 Gaucher Disease patients regardless of imiglucerase-antibody status, as inclusion of these patients would more accurately represent the expected clinical use of GA-GCB, and would support the use of GA-GCB in a broader patient population.
21. The stopping rules for your study (in protocol section 9.4) state that "If any patient experiences a life-threatening (Grade 4) serious adverse event (SAE), or death occurs that is considered possibly or probably related to the study drug, the decision to stop the study requires agreement by the Shire HGT Medical Monitor, the Investigator, and the IRB/IEC." Please revise the stopping rules for the study based on specific safety criteria, rather than on the subjective assessment of events by study personnel.
22. The procedures for Serious Adverse Event (SAE) reporting (in protocol section 9.3.1) state that "Any SAE that occurs after administration of the first dose of GA-GCB must be reported in the event of a severe, possibly or probably related AE or SAE..." Please revise the SAE reporting procedures in your protocol to more accurately reflect the requirements under 21 CFR 312.32(c)(1) and (2), whereby "The sponsor shall notify FDA and all participating investigators in a written IND safety report of: (A) Any adverse experience associated with the use of the drug that is both serious and

IND 61,220
Page 6

unexpected..." and "The sponsor shall also notify FDA by telephone or by facsimile transmission of any unexpected fatal or life-threatening experience associated with the use of the drug... in no event later than 7 calendar days..." Please note that this requirement does not include a subjective assessment (possibly or probably related) of the event.

23. Please revise your protocol so that all pediatric patients participating in the study are to undergo assessments of growth at regular intervals in the study. Assessments of growth including, at minimum, assessments of height and weight, are to be obtained in a standardized manner that are to be delineated in the study protocol (e.g., height measured via a calibrated stadiometer, and the final measurement taken as an average of three measurements).
24. Your sample Informed Consent form states (on page 5, paragraph 7) that children as young as two years of age will be undergoing magnetic resonance imaging (MRI) testing. In young children, sedation is often required for MRI testing. We recommend that you revise your sample Informed Consent form to note the possible need for sedation in pediatric patients for MRI testing, and an explanation of the risks of sedation in these patients.

Please cite the IND number listed above at the top of the first page of any communications concerning this application. Send all submissions, electronic or paper, including those sent by overnight mail or courier, to the following address:

Food and Drug Administration
Center for Drug Evaluation and Research
Division of Gastroenterology
5901-B Ammendale Road
Beltsville, MD 20705-1266

If you have any questions, call Ryan Barraco, Regulatory Project Manager, at (301) 796-0846.

Sincerely,

{See appended electronic signature page}

Brian E. Harvey, M.D., Ph.D.
Director
Division of Gastroenterology Products
Office of Drug Evaluation III
Center for Drug Evaluation and Research

**This is a representation of an electronic record that was signed electronically and
this page is the manifestation of the electronic signature.**

/s/

Brian Harvey

11/27/2006 04:00:35 PM

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment I

Letter from FDA to Shire Human Genetic Therapies, Inc., dated December 21, 2006, removing the clinical hold and indicating that the protocol can be initiated



DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Rockville, MD 20857

IND 61,220

Shire Human Genetic Therapies
Attention: Nikhil Mehta, Ph.D.
Vice President, Global Regulatory Affairs
700 Main Street
Cambridge, MA 02139

REGULATORY AFFAIRS
RECEIVED

REGULATORY AFFAIRS
RECEIVED

JAN 5 2003

SHIRE HGT

SHIRE HGT

Dear Dr. Mehta:

Please refer to your Investigational New Drug Application (IND) submitted December 30, 2003, under section 505(i) of the Federal Food, Drug, and Cosmetic Act for Gene Activated Glucocerebrosidase (GA-GCB).

We also refer to your amendment dated November 30, 2006 (serial # 041), which provided a complete response to our December 7, 2006, letter which cited the reasons for placing Protocol TKT032, titled "A Multi-center, Randomized, Double-Blind, Parallel Group, Two-Dose Study of Gene-Activated™ Human Glucocerebrosidase (GAGCB) Enzyme Replacement Therapy in Patients with Type I Gaucher Disease," on clinical hold and the information needed to resolve the clinical hold issues.

We have completed the review of your submission, and have concluded that the above protocol may be initiated.

We have the following comments, however, regarding your clinical development program:

A direct comparison of IEX-HPLC and glycan mapping data for drug substance (DS) lots E303-005, E303-006, E303-007, and EP06-003 indicate that physico-chemical differences exist between the DS manufactured with different processes that were used in clinical trials. However, these differences do not appear to pose a serious safety risk to human subjects, and clinical trials with the Animal Free (AF) DS appear to be safe to proceed at this time. Nevertheless, in view of these differences, please be aware that you might not be able to use the Phase 1 clinical data generated using the serum-containing DS to support a future marketing application for GA-GCB.

As sponsor of this IND, you are responsible for compliance with the Federal Food, Drug, and Cosmetic Act and the implementing regulations (Title 21 of the Code of Federal Regulations). Those responsibilities include (1) reporting any unexpected fatal or life-threatening adverse experience associated with use of the drug by telephone or fax no later than 7 calendar days after initial receipt of the information [21 CFR 312.32(c)(2)]; (2) reporting any adverse experience associated with use of the drug that is both serious and unexpected in writing no later than 15 calendar days after initial receipt of the information [21 CFR 312.32(c)(1)]; and (3) submitting annual progress reports [21 CFR 312.33].

IND 61,220
Page 2

If you have any questions, call Ryan Barraco, Regulatory Project Manager, at (301) 796-0846.

Sincerely,

{See appended electronic signature page}

Brian E. Harvey, M.D., Ph.D.
Director
Division of Gastroenterology Products
Office of Drug Evaluation III
Center for Drug Evaluation and Research

REGULATORY AFFAIRS
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JAN 5 2007

SHIRE HGT

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/s/

Brian Harvey

12/21/2006 05:11:30 PM

In re U.S. Patent No.: 7,138,262 B1
Issued: November 21, 2006
Inventors: Peter Francis Daniel
Assignee: Shire Human Genetic Therapies,
Inc.

Attorney Docket No.: S2071-701019/0013US

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment J

Letter from FDA to Shire Human Genetic Therapies, Inc., dated September 14,
2009, acknowledging receipt of the final submission of the NDA



DEPARTMENT OF HEALTH AND HUMAN SERVICES

Food and Drug Administration
Silver Spring MD 20993

NDA 22575

Shire Human Genetic Therapies, Inc.
Attention: Nikhil Mehta, Ph.D.
Vice President, Global Regulatory Affairs
700 Main Street
Cambridge, MA 02139

NDA ACKNOWLEDGMENT

SHIRE HGT
Regulatory Affairs

SEP 14 2009

Received

Dear Dr. Mehta:

We have received your new drug application (NDA) submitted under section 505(b) of the Federal Food, Drug, and Cosmetic Act (FDCA) for the following:

Name of Drug Product: TRADENAME (velagluferase alfa)

Date of Application: August 31, 2009

Date of Receipt: August 31, 2009

Our Reference Number: NDA 22575

Unless we notify you within 60 days of the receipt date that the application is not sufficiently complete to permit a substantive review, we will file the application on October 30, 2009, in accordance with 21 CFR 314.101(a).

If you have not already done so, promptly submit the content of labeling [21 CFR 314.50(l)(1)(i)] in structured product labeling (SPL) format as described at <http://www.fda.gov/oc/datacouncil/spl.html>. Failure to submit the content of labeling in SPL format may result in a refusal-to-file action under 21 CFR 314.101(d)(3). The content of labeling must conform to the content and format requirements of revised 21 CFR 201.56-57.

The NDA number provided above should be cited at the top of the first page of all submissions to this application. Send all submissions, electronic or paper, including those sent by overnight mail or courier, to the following address:

Food and Drug Administration
Center for Drug Evaluation and Research
Division of Gastroenterology Products
5901-B Ammendale Road
Beltsville, MD 20705-1266

All regulatory documents submitted in paper should be three-hole punched on the left side of the page and bound. The left margin should be at least three-fourths of an inch to assure text is not obscured in the fastened area. Standard paper size (8-1/2 by 11 inches) should be used; however, it may occasionally be necessary to use individual pages larger than standard paper size.

Non-standard, large pages should be folded and mounted to allow the page to be opened for review without disassembling the jacket and refolded without damage when the volume is shelved. Shipping unbound documents may result in the loss of portions of the submission or an unnecessary delay in processing which could have an adverse impact on the review of the submission. For additional information, please see <http://www.fda.gov/cder/ddms/binders.htm>.

If you have any questions, call me at (301) 796-0069.

Sincerely,

{See appended electronic signature page}

R. Wesley Ishihara
Regulatory Health Project Manager
Division of Gastroenterology Products
Office of Drug Evaluation III
Center for Drug Evaluation and Research

Application
Type/Number

Submission
Type/Number

Submitter Name

Product Name

NDA-22575

ORIG-1

SHIRE HGT INC.

VELAGLUCERASE ALFA

This is a representation of an electronic record that was signed electronically and this page is the manifestation of the electronic signature.

/s/

RICHARD W ISHIHARA

09/14/2009

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment K

Certification of Copies of Application Papers

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re U.S. Patent No.: 7,138,262 B1

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

CERTIFICATE OF EXPRESS MAILING UNDER 37 C.F.R. §1.10

The undersigned hereby certifies that this document was deposited with the U.S. Postal Service on April 22, 2010 for express mailing in accordance with §1.6(a)(2).


Laurie Butler Lawrence, Reg. No. 46,593

Mail Stop Hatch-Waxman PTE

Commissioner for Patents

P.O. Box 1450

Alexandria, VA 22313-1450

APPLICATION FOR EXTENSION OF PATENT TERM UNDER 35 U.S.C. § 156

Applicant, Shire Human Genetic Therapies, Inc. represents that it is the Assignee of the entire interest in and to United States Patent No. 7,138,262 B1 granted to Shire Human Genetic Therapies, Inc. on the 21st day of November 2006, for "High Mannose Proteins and Methods of Making High Mannose Proteins" by virtue of an assignment from Peter Francis Daniel to Transkaryotic Therapies, Inc., recorded in the U.S. Patent and Trademark Office at Reel 011662, Frame 0815, on March 28, 2001, and from Transkaryotic Therapies, Inc. to Shire Human Genetic Therapies, Inc., recorded in the U.S. Patent and Trademark Office at Reel 018224, Frame 0390, on August 31, 2006.

By the Power of Attorney enclosed herein (Attachment A), Applicant has appointed several individual attorneys, including Laurie Butler Lawrence, as attorneys for Shire Human Genetic Therapies, Inc. with regard to this application for extension of the term of U.S. Patent No. 7,138,262 B1 and to transact all business in the U.S. Patent and Trademark Office in connection therewith.

In re U.S. Patent No.: 7,138,262 B1
Issued: November 21, 2006
Inventors: Peter Francis Daniel
Assignee: Shire Human Genetic Therapies,
Inc.
Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attorney Docket No.: S2071-701019/0013US

Transkaryotic Therapies, Inc. became Shire Human Genetic Therapies, Inc. on January 17, 2006. Shire Human Genetic Therapies, Inc. is the holder of the regulatory approval granted with respect to the regulatory review period relied on herein.

Information Required Under 37 C.F.R. § 1.740

Applicant hereby submits this application for extension of the patent term under 35 U.S.C. § 156 by providing the following information required by the rules promulgated by the U.S. Patent and Trademark Office (37 C.F.R. § 1.740). For the convenience of the Patent and Trademark Office, the information contained in this application will be presented herein in a format which follows the order of the requirements of Section 1.740 of Title 37 of the Code of Federal Regulations.

(1) Identification of the Approved Product [1.740(a)(1)]

The approved product is VPRIVTM. The name of the active ingredient in VPRIVTM is velaglucerase alfa for injection. Velaglucerase alfa for injection is a hydrolytic lysosomal glucocerebroside-specific enzyme indicated for long-term replacement therapy (ERT) for pediatric and adult patients with type 1 Gaucher disease. The active ingredient of VPRIVTM is velaglucerase alfa, which is produced by gene activation technology in a human fibroblast cell line. Velaglucerase alfa is a glycoprotein of 497 amino acids; with a molecular weight of approximately 63 kDa. Velaglucerase alfa has the same amino acid sequence as the naturally occurring human enzyme, β -glucocerebrosidase. Velaglucerase alfa contains 5 potential N-linked glycosylation sites; four of these sites are occupied by glycan chains. Velaglucerase alfa is manufactured to contain predominantly high mannose-type N-linked glycan chains. VPRIVTM is supplied as a sterile, preservative free, lyophilized powder in single-use vials. Following reconstitution with Sterile Water for Injection, USP, the solution contains the components listed in Table 3 of the package insert, which is provided in Attachment B (a copy of the

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

package insert is also provided as an enclosure to the NDA approval letter from FDA to Shire Human Genetic Therapies, Inc., dated February 26, 2010 in Attachment C).

(2) Federal Statute Governing Regulatory Approval of the Approved Product [1.740(a)(2)]

The approved product, VPRIVTM, was subject to regulatory review under § 505(i) and §505(b) of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. § 355(i) and § 355(b)).

(3) Date of Approval for Commercial Marketing [1.740(a)(3)]

The approved product, VPRIVTM, received permission for commercial marketing or use under Section 505(b) of the Federal Food, Drug, and Cosmetic Act on February 26, 2010. A copy of the NDA approval letter from FDA to Shire Human Genetic Therapies, Inc., dated February 26, 2010 (with enclosure), is provided as Attachment C.

(4) Identification of Active Ingredient and Certifications Related to Commercial Marketing of Approved Product [1.740(a)(4)]

The only active ingredient in VPRIVTM is velaglucerase alfa for injection which, on information and belief, has not been previously approved for commercial marketing or use under the Public Health Service Act, the Virus-Serum-Toxin Act or the Federal Food, Drug, and Cosmetic Act. A copy of the package insert describing the approved product is attached (Attachment B).

In re U.S. Patent No.: 7,138,262 B1
Issued: November 21, 2006
Inventors: Peter Francis Daniel
Assignee: Shire Human Genetic Therapies,
Inc.

Attorney Docket No.: S2071-701019/0013US

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

**(5) Statement Regarding Timeliness of Submission of Patent Term
Extension Request [1.740(a)(5)]**

This application for extension of patent term under 35 U.S.C. § 156 is being submitted within the permitted 60-day period pursuant to 37 C.F.R. § 1.720(f). The last day on which this application can be submitted is April 26, 2010.

**(6) Complete Identification of the Patent for Which Extension Is Being
Sought [1.740(a)(6)]**

The complete identification of the patent for which a term extension is being sought is as follows:

Inventors:	Peter Francis Daniel
Patent No.:	7,138,262 B1
Filing Date:	August 18, 2000
Issue Date:	November 21, 2006
Expiration Date:	August 18, 2020

**(7) Copies of the Patent for Which an Extension is Being Sought
[1.740(a)(7)]**

A copy of U.S. Patent No.: 7,138,262 B1 is provided as Attachment D.

**(8) Copies of Disclaimers, Certificates of Correction, Receipt of
Maintenance Fee Payments, or Reexamination Certificate [1.740(a)(8)]**

- (a) U.S. Patent No.: 7,138,262 B1 is not subject to a terminal disclaimer.
- (b) No certificate of correction has been issued for U.S. Patent No.: 7,138,262 B1.
- (c) The first maintenance fee for U.S. Patent No.: 7,138,262 B1 will be due with a payment of the surcharge on May 22, 2010. This maintenance fee has been paid as

In re U.S. Patent No.: 7,138,262 B1
Issued: November 21, 2006
Inventors: Peter Francis Daniel
Assignee: Shire Human Genetic Therapies,
Inc.

Attorney Docket No.: S2071-701019/0013US

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

shown in the copy of the USPTO's on-line record of patent maintenance fee payment for this patent which is attached (Attachment E).

(d) U.S. Patent No.: 7,138,262 B1 has not been the subject of a reexamination proceeding.

(9) Statement Regarding Patent Claims Relative to Approved Product
[1.740(a)(9)]

The following claims of U.S. Patent No. 7,138,262 B1 claim a method of manufacturing the approved product, VPRIVTM: claims 1-4, 12-18, 23, 26-38, 48-54, and 57-61.

(iii) Pursuant to M.P.E.P. § 2753 and 37 C.F.R. § 1.740(a)(9)(iii), the following explanation is provided which demonstrates the manner in which at least one such patent claim reads on the method of manufacturing the approved product, VPRIVTM.

Description of the approved product and the method of manufacturing the same:

Velaglucerase alfa for injection is human β -glucocerebrosidase produced by gene-activation in immortalized human fibroblast HT-1080 cells. Gene activation refers to the introduction of an exogenous promoter into the cell that activates the endogenous human β -glucocerebrosidase gene. The activated gene expresses human β -glucocerebrosidase. β -glucocerebrosidase has 5 potential N-linked glycosylation sites, four of which are occupied by glycan chains in velaglucerase alfa for injection.

Glycosylation of velaglucerase alfa for injection is altered by culturing the cells in the presence of kifunensine, a mannosidase I inhibitor, at 2 μ g/ml. This results in the secretion of human β -glucocerebrosidase containing primarily high-mannose type glycan chains having 6-9 mannose units per glycan chain. The cells are cultured under conditions wherein:

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the mannosidase inhibitor prevents removal of one or more α 1,2 mannose residue(s) distal to the pentasaccharide core; the removal of one or more mannose residues distal to the pentasaccharide core is prevented on at least two carbohydrate chains of hmGCB; at least 60% of the high mannose glucocerebrosidase (hmGCB) of the preparation have three or more carbohydrate chains in which the removal of one or more mannose residues distal to the pentasaccharide core has been prevented; at least about 60% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues; the mannosidase inhibitor further prevents the removal of one α 1,3 mannose residue distal to the pentasaccharide core; the mannosidase inhibitor further prevents the removal of one α 1,6 mannose residue distal to the pentasaccharide core; the mannosidase inhibitor prevents removal of at least three mannose residues distal to the pentasaccharide core of the precursor oligosaccharide of GCB; at least 60% of the hmGCB of the preparation have one or more carbohydrate chains in which the removal of three or more mannose residues distal to the pentasaccharide core has been prevented; at least 60% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues; and at least about 80% or more of the carbohydrate chains of the hmGCB preparation have six or more mannose residues.

Velaglucerase alfa for injection is harvested from media in which the cells are cultured.

Velaglucerase alfa for injection is secreted as a monomeric glycoprotein of approximately 63 kDa and is composed of 497 amino acids with a sequence identical to the natural human protein. The amino acid sequence of velaglucerase alfa for injection is described in Zimran et al. (2007) *Blood Cells Mol Dis*, 39: 115-118. Velaglucerase alfa for injection contains 5 potential N-linked glycosylation sites; four of these sites are occupied by glycan chains.

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A summary of the glycan structure, and other aspects of velaglucerase alfa for injection, is provided in Brumshtein et al. (2010) Glycobiology 20(1):24-32 as Attachment F. See, e.g., Table III, therein.

As is discussed below, claims 1-4, 12-18, 23, 26-38, 48-54, and 57-61 of U.S. Patent No. 7,138,262 B1 read on the method of manufacturing the approved product. The claims are set out in the left hand column of the table immediately below. The method of manufacturing the approved product is described in the right hand column and compared with the claim. As is shown, the approved product meets all of the limitations of each of claims 1-4, 12-18, 23, 26-38, 48-54, and 57-61 and claims 1-4, 12-18, 23, 26-38, 48-54, and 57-61 cover the method of manufacturing the approved product, VPRIV™.

<p>1. A method of producing a preparation of high mannose glucocerebrosidase (hmGCB) comprising a carbohydrate chain having at least four mannose residues,</p> <p>comprising: providing a mammalian cell that expresses a human glucocerebrosidase (GCB);</p>	<p>VPRIV™ includes at least two glucocerebrosidase (GCB) proteins that have at least one carbohydrate chain having four or more mannose residues. See, e.g., page 24, column 1 of Attachment F, “the predominant glycan on velaglucerase alfa is a high mannose type, with nine mannose residues”. See also Table III and Figures 7 and 8 of Attachment F. See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>VPRIV™ is produced in a human cell line that expresses human glucocerebrosidase See page 24, column 2 of Attachment F: “we have used gene activation in a well characterized, continuous human cell line to produce gene activated human acid-β-glucocerebrosidase (velaglucerase alfa).” See also the description of the approved product and the method of manufacturing</p>
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<p>contacting the cell with kifunensine; allowing the cell to produce hmGCB; and</p> <p>harvesting the hmGCB from the cell or its culture media, to thereby produce an hmGCB preparation.</p>	<p>the same in this section (9)(iii).</p> <p>The human cell line expressing VPRIVTM is contacted with kifunensine and the cells secrete VPRIV. See page 24, column 2 of Attachment F, "glycosylation of velaglycerase alfa is altered by using kifunensine ... during cell culture, which results in the secretion of a protein containing predominantly high-mannose type glycans" See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>VPRIVTM is harvested from the cell culture to produce a preparation with at least two glucocerebrosidase proteins having at least one carbohydrate chain having four or more mannose residues. See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 1 and the claim covers the method of manufacturing the approved product.</p>
<p>2. The method of claim 1, wherein removal of one or more α1,2 mannose residue(s) distal to the pentasaccharide core is prevented.</p>	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>VPRIVTM includes at least 2 GCB proteins that have one or more mannose residue distal to the pentasaccharide core present. See the description of the approved product and the method of manufacturing the same</p>

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	<p>in this section (9)(iii).</p> <p>Therefore, the method of making VPRIV meets all of the limitations of claim 2 and the claim covers the method of manufacturing the approved product.</p>
<p>3. The method of claim 1, wherein the kifunensine is present at a concentration between about 0.05 to 20.0 µg/ml.</p>	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>Kifunensine is present at a concentration 2.0 µg/ml in the method of making the approved product. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 3 and the claim covers the method of manufacturing the approved product.</p>
<p>4. The method of claim 3, wherein the kifunensine is present at a concentration between about 0.1 to 2.0 µg/ml.</p>	<p>As discussed above for claims 1 and 3, the method of making the approved product meets all of the limitations of the base claims.</p> <p>Kifunensine is present at a concentration of 2.0 µg/ml in the method of making the approved product. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM</p>

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	meets all of the limitations of claim 4 and the claim covers the method of making the approved product.
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12. The method of claim 1, wherein the removal of one or more mannose residues distal to the pentasaccharide core is prevented on at least two carbohydrate chains of hmGCB.	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>Kifunensine is an inhibitor of endoplasmic reticulum mannosidase I and Golgi mannosidase I enzymes (see column 23, lines 48-51 of the '262 patent) that prevents the removal of one or more mannose residues distal to the pentasaccharide core. (see, e.g., Fig. 7 of Attachment F which shows the predominant glycan structure present on the GCB in VPRIVTM). See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 12 and the claim covers the method of manufacturing the approved product.</p>
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13. The method of claim 1, wherein at least 60% of the hmGCB of the preparation have one or more carbohydrate chains in which the removal of one or more mannose residues distal to the pentasaccharide core has been prevented.	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>At least 60% of the hmGCB of the preparation have one or more carbohydrate chains in which the removal of one or more</p>
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	<p>mannose residues distal to the pentasaccharide core has been prevented. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIV™ meets all of the limitations of claim 13 and the claim covers the method of manufacturing the approved product.</p>
14. The method of claim 13, wherein the removal of three or more mannose residues distal to the pentasaccharide core has been prevented.	<p>As discussed above for claims 1 and 13, the method of making the approved product meets all of the limitations of the base claims.</p> <p>The removal of three or more mannose residues distal to the pentasaccharide core has been prevented. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIV™ meets all of the limitations of claim 14 and the claim covers the method of manufacturing the approved product.</p>
15. The method of claim 1, wherein at least about 20% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>At least about 20% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues. See the description of the</p>

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	<p>approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIV™ meets all of the limitations of claim 15 and the claim covers the method of manufacturing the approved product.</p>
<p>16. The method of claim 15, wherein at least about 40% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.</p>	<p>As discussed above for claims 1 and 15, the method of making the approved product meets all of the limitations of the base claim.</p> <p>At least about 40% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIV™ meets all of the limitations of claim 16 and the claim covers the method of manufacturing the approved product.</p>

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<p>17. The method of claim 16, wherein at least about 60% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.</p>	<p>As discussed above for claims 1, 15 and 16, the method of making the approved product meets all of the limitations of the base claims.</p> <p>At least about 60% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 17 and the claim covers the method of manufacturing the approved product.</p>
<p>18. The method of claim 1, wherein at least about 80% or more of the carbohydrate chains of the hmGCB preparation have six or more mannose residues.</p>	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>At least about 80% or more of the carbohydrate chains of the hmGCB preparation have six or more mannose residues. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 18 and the claim covers the method of manufacturing the approved product.</p>

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<p>23. The method of claim 1, wherein the cell comprises an exogenous regulatory sequence which functions to regulate expression of an endogenous GCB coding sequence.</p>	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>VPRIVTM is produced by a human cell line that comprises an exogenous promoter sequence to regulate expression of an endogenous GCB coding sequence. (see, e.g., page 24, column 2 of Attachment F- “we have used gene activation in a well-characterized, continuous human cell line to produce gene activated human acid-β-glucocerebrosidase (velaglucerase alfa). Gene activation refers to targeted recombination with a promoter that activates the endogenous GlcCerase gene in the selected human cell line.” See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 23 and the claim covers the method of manufacturing the approved product.</p>
<p>26. The method of claim 1, wherein the cell is a human cell.</p>	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>VPRIVTM is produced by a human cell line. See page 24, column 2 of Attachment F – “we have used gene activation in a well characterized, continuous human cell line to produce gene activated human acid-β-glucocerebrosidase (velaglucerase alfa).” See also the description of the approved product and the method of manufacturing</p>

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	<p>the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 26 and the claim covers the method of manufacturing the approved product.</p>
27. The method of claim 26, wherein the cell is a fibroblast or a myoblast.	<p>As discussed above for claims 1 and 26, the method of making the approved product meets all of the limitations of the base claims.</p> <p>VPRIVTM is produced by HT-1080 cells which are fibroblasts. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 27 and the claim covers the method of manufacturing the approved product.</p>
28. The method of claim 26, wherein the cell is an immortalized cell.	<p>As discussed above for claims 1, 26 and 27, the method of making the approved product meets all of the limitations of the base claims.</p> <p>VPRIVTM is produced by an immortalized cell. See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 28 and the claim covers the method of making the</p>

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	approved product.
29. The method of claim 27, wherein the cell is an HT-1080 cell.	<p>As discussed above for claims 1, 26, 27 and 28, the method of making the approved product meets all of the limitations of the base claims.</p> <p>VPRIVTM is produced by HT-1080 cells. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 29 and the claim covers the method of manufacturing the approved product.</p>
30. The method of claim 1, wherein the cell is contacted with kifunensine in culture media.	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>VPRIVTM is produced by contacting the cells expressing hGCB with kifunensine in cell culture. See page 24, column 2 of Attachment F, "glycosylation of velaglucerase alfa is altered by using kifunensine ... during cell culture, which results in the secretion of a protein containing predominantly high-mannose type glycans." See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 30 and the claim covers the method of manufacturing the approved product.</p>

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31. The method of claim 30, wherein the hmGCB is obtained from the media in which the cell is cultured.	<p>As discussed above for claims 1 and 30, the method of making the approved product meets all of the limitations of the base claims.</p> <p>VPRIVTM is obtained from the media in which the human cell line is cultured. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 31 and the claim covers the method of manufacturing the approved product.</p>

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<p>32. A method of producing a preparation of high mannose glucocerebrosidase (hmGCB) comprising a carbohydrate chain having at least four mannose residues, the method comprising:</p> <p>providing a human cell into which a nucleic acid sequence comprising an exogenous regulatory sequence has been introduced such that the regulatory sequence is operably linked to, and regulates the expression of, an endogenous GCB coding region;</p> <p>contacting the cell with a class 1 mannosidase inhibitor such that the removal of at least one mannose residue distal to the pentasaccharide core of a precursor oligosaccharide of GCB is prevented; and allowing the cell to produce hmGCB, to thereby produce an hmGCB preparation.</p>	<p>VPRIVTM includes at least two glucocerebrosidase (GCB) proteins having at least one carbohydrate chain having four or more mannose residues. See, e.g., page 24, column 1 of Attachment F, “the predominant glycan on velaglucerase alfa is a high mannose type, with nine mannose residues”. See also Table III and Figures 7 and 8 of Attachment F. See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>VPRIVTM is produced by a human cell that includes an exogenous promoter sequence to regulate expression of an endogenous GCB coding sequence. (see, e.g., page 24, column 2 of Attachment F-“we have used gene activation in a well-characterized, continuous human cell line to produce gene activated human acid-β-glucocerebrosidase (velaglucerase alfa). Gene activation refers to targeted recombination with a promoter that activates the endogenous GlcCerase gene in the selected human cell line.” See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>VPRIVTM is produced by contacting the cells expressing hGCB with kifunensine, which is a class 1 mannosidase inhibitor, in cell culture. See page 24, column 2 of Attachment F, “glycosylation of velaglucerase alfa is altered by using kifunensine ... during cell culture, which results in the secretion of a protein</p>
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	<p>containing predominantly high-mannose type glycans". VPRIVTM includes at least 2 GCB proteins having at least one carbohydrate chain with at least one mannose residue distal to the pentasaccharide core, e.g., the GCB proteins have four or more mannose residues. See, e.g., Table III of Attachment F. See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 32 and the claim covers the method of manufacturing the approved product.</p>
<p>33. The method of claim 32, wherein the mannosidase inhibitor prevents the removal of one or more α1,2 mannose residue(s) distal to the pentasaccharide core.</p>	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>The mannosidase inhibitor prevents the removal of one or more α1,2 mannose residue(s) distal to the pentasaccharide core. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 33 and the claim covers the method of manufacturing the approved product.</p>
<p>34. The method of claim 32, wherein the mannosidase inhibitor further prevents the</p>	<p>As discussed above for claim 32, the method of making the approved product</p>

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<p>removal of one α1,3 mannose residue distal to the pentasaccharide core.</p>	<p>meets all of the limitations of the base claim.</p> <p>The mannosidase inhibitor further prevents the removal of one α1,3 mannose residue distal to the pentasaccharide core. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 34 and the claim covers the method of manufacturing the approved product.</p>
<p>35. The method of claim 32, wherein the mannosidase inhibitor further prevents the removal of one α1,6 mannose residue distal to the pentasaccharide core.</p>	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>The mannosidase inhibitor further prevents the removal of one α1,6 mannose residue distal to the pentasaccharide core. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 35 and the claim covers the method of manufacturing the approved product.</p>
<p>36. The method of claim 32, wherein the class 1 processing mannosidase inhibitor is kifunensine.</p>	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p>

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	<p>VPRIVTM is produced by contacting the cells expressing hGCB with kifunensine in cell culture. See page 24, column 2 of Attachment F, "glycosylation of velaglucerase alfa is altered by using kifunensine ... during cell culture, which results in the secretion of a protein containing predominantly high-mannose type glycans". See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 36 and the claim covers the method of manufacturing the approved product.</p>
37. The method of claim 36, wherein the kifunensine is present at a concentration between about 0.05 to 20.0 µg/ml.	<p>As discussed above for claims 32 and 36, the method of making the approved product meets all of the limitations of the base claims.</p> <p>Kifunensine is present at a concentration 2.0 µg/ml in the method of making the approved product. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 37 and the claim covers the method of manufacturing the approved product.</p>

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<p>38. The method of claim 37, wherein the kifunensine is present at a concentration between about 0.1 to 2.0 µg/ml.</p>	<p>As discussed above for claims 32, 36 and 37, the method of making the approved product meets all of the limitations of the base claims.</p> <p>Kifunensine is present at a concentration 2.0 µg/ml in the method of making the approved product. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 38 and the claim covers the method of manufacturing the approved product.</p>
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<p>48. The method of claim 32, wherein the mannosidase inhibitor prevents removal of at least three mannose residues distal to the pentasaccharide core of the precursor oligosaccharide of GCB.</p>	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>Kifunensine is an inhibitor of endoplasmic reticulum mannosidase I and Golgi mannosidase I enzymes (see column 23, lines 48-51 of the '262 patent) that prevents the removal of three or more mannose residues distal to the pentasaccharide core. (see, e.g., Fig. 7 of Attachment F which shows the predominant glycan structure present on the GCB in VPRIVTM). See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p>
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	Therefore, the method of making VPRIV TM meets all of the limitations of claim 48 and the claim covers the method of manufacturing the approved product.
49. The method of claim 32, wherein the mannosidase inhibitor prevents removal of one or more mannose residues distal to the pentasaccharide core on at least two of the carbohydrate chains of hmGCB.	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>Kifunensine is an inhibitor of endoplasmic reticulum mannosidase I and Golgi mannosidase I enzymes (see column 23, lines 48-51 of the '262 patent) that prevents the removal of one or more mannose residues distal to the pentasaccharide core. (see, e.g., Fig. 7 of Attachment F which shows the predominant glycan structure present on the GCB in VPRIVTM). See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 49 and the claim covers the method of manufacturing the approved product.</p>
50. The method of claim 32, wherein at least 60% of the hmGCB of the preparation have one or more carbohydrate chains in which the removal of three or more mannose residues distal to the pentasaccharide core has been prevented.	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>At least 60% of the hmGCB of the preparation have one or more carbohydrate</p>

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	<p>chains in which the removal of three or more mannose residues distal to the pentasaccharide core has been prevented. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 50 and the claim covers the method of manufacturing the approved product.</p>
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51. The method of claim 32, wherein at least 20% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>At least 20% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 51 and the claim covers the method of manufacturing the approved product.</p>
52. The method of claim 51, wherein at least 40% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.	<p>As discussed above for claims 32 and 51, the method of making the approved product meets all of the limitations of the base claims.</p>

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	<p>At least 40% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 52 and the claim covers the method of manufacturing the approved product.</p>
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53. The method of claim 52, wherein at least 60% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.	<p>As discussed above for claims 32, 51 and 52, the method of making the approved product meets all of the limitations of the base claims.</p> <p>At least 60% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 53 and the claim covers the method of manufacturing the approved product.</p>
54. The method of claim 32, wherein at least about 80% or more of the carbohydrate chains of the hmGCB preparation have six or more mannose residues.	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>At least about 80% or more of the</p>

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	<p>carbohydrate chains of the hmGCB preparation have six or more mannose residues. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 54 and the claim covers the method of manufacturing the approved product.</p>
57. The method of claim 32, wherein the cell is a fibroblast or a myoblast.	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>VPRIVTM is produced by an HT-1080 cell line, which is a fibroblast. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 57 and the claim covers the method of manufacturing the approved product.</p>
58. The method of claim 32, wherein the cell is an immortalized cell.	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>VPRIVTM is produced by an immortalized cell. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p>

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	Therefore, the method of making VPRIV™ meets all of the limitations of claim 58 and the claim covers the method of making the approved product.
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59. The method of claim 58, wherein the cell is an HT-1080 cell.	<p>As discussed above for claims 32 and 58, the method of making the approved product meets all of the limitations of the base claims.</p> <p>VPRIV™ is produced by an HT-1080 cell line. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIV™ meets all of the limitations of claim 59 and the claim covers the method of manufacturing the approved product.</p>
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<p>60. The method of claim 36, wherein the cell is contacted with kifunensine in culture media.</p>	<p>As discussed above for claims 32 and 36, the method of making the approved product meets all of the limitations of the base claims.</p> <p>VPRIVTM is produced by contacting the cells expressing hGCB with kifunensine in cell culture. See page 24, column 2 of Attachment F, "glycosylation of velaglucerase alfa is altered by using kifunensine ... during cell culture, which results in the secretion of a protein containing predominantly high-mannose type glycans" See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 60 and the claim covers the method of manufacturing the approved product.</p>
<p>61. The method of claim 60, wherein the hmGCB is obtained from the media in which the cell is cultured.</p>	<p>As discussed above for claims 32, 36, and 60, the method of making the approved product meets all of the limitations of the base claims.</p> <p>VPRIVTM is obtained from the media that the cell line is cultured. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 61 and the claim covers the method of</p>

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	manufacturing the approved product.
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**(10) Relevant Dates Under 35 U.S.C. § 156 for Determination of
Applicable Regulatory Review Period [1.740(a)(10)]**

The relevant dates and information pursuant to 35 U.S.C. § 156(g) to enable the Secretary of Health and Human Services to determine the applicable regulatory review period are as follows:

Patent Issue Date:

U.S. Patent No. 7,138,262 B1 issued on November 21, 2006.

***(i)(A) IND Effective Date and IND number [35 U.S.C. §156(g)(1)(B)(i); 37
C.F.R. §1.740(a)(10)(i)(A)]***

The effective date of IND 61,220 was May 20, 2004.

An IND was by submitted by Transkaryotic Therapies, Inc. to FDA and received by FDA on December 31, 2003. It was assigned number IND 61,220. A copy of the letter from FDA to Transkaryotic Therapies, Inc., dated January 12, 2004, providing the IND number and showing the date of receipt by FDA of the IND is provided in Attachment G. On January 28, 2004, FDA notified Transkaryotic Therapies, Inc. that a modification to the protocol was necessary. A written record of the discussion is provided in Attachment G1. Transkaryotic Therapies, Inc submitted an amendment to the protocol on March 11, 2004, see letter from Transkaryotic Therapies, Inc. to FDA, dated March 11, 2004, concerning amendment of protocol, provided in Attachment G2. On May 20, 2004, FDA notified Transkaryotic Therapies, Inc. that it could proceed, see FDA communication to Transkaryotic Therapies, Inc. dated May 20, 2004, concerning amendment to protocol, a copy of which is provided in Attachment G3.

On November 20, 2006, FDA notified Shire Human Genetics Therapies, Inc. that IND 61,220 was on clinical hold. A copy of the letter from the FDA to Shire Human Genetics Therapies, Inc. showing the date the FDA notified Shire Human Genetics

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Therapies, Inc. of the clinical hold is provided as Attachment H. The FDA removed the clinical hold on December 21, 2006. A copy of the letter dated December 21, 2006 from the FDA to Shire Human Genetics Therapies, Inc. indicating that the hold had been removed and the protocol could be initiated is provided as Attachment I.

Thus, as set out above, the date that an exemption under §505(i) of the Federal Food, Drug and Cosmetic Act became effective (i.e., the date that an investigational new drug application (IND) became effective for VPRIV™) was May 20, 2004.

(i)(B) NDA Submission Date [35 U.S.C. §156(g)(1)(B)(i); 37 C.F.R.

§1.740(a)(10)(i)(B)] The NDA was submitted on a rolling basis. The initial portion of the NDA was submitted by Shire Human Genetic Therapies, Inc. to the FDA on July 30, 2009. The final portion was submitted on August 31, 2009. This date is used in the calculations provided herein. The NDA was assigned number NDA 22575. A copy of a letter from FDA to Shire Human Genetic Therapies, Inc., dated September 14, 2009, acknowledging receipt of the final submission of the NDA application is provided as Attachment J.

(i)(C) NDA Approval Date [35 U.S.C. §156(g)(1)(B)(ii); 37 C.F.R.

§1.740(a)(10)(i)(C)]

The FDA approved NDA 22575 authorizing the marketing of VPRIV™ on February 26, 2010. VPRIV™ was approved under the Department of Health and Human Services (DHHS). A copy of the approval letter from FDA to Shire Human Genetic Therapies, Inc., dated February 26, 2009 is provided as Attachment C.

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(11) Summary of Significant Events During Regulatory Review Period

[1.740(a)(11)]

A brief description of the significant activities undertaken by the marketing applicant during the applicable regulatory review period with respect to VPRIVTM and the dates applicable to these significant activities are set forth in a chronology of events provided below.

Date	IND or NDA Serial No. (if applicable)	Other Contact	Description
06 April 2001			Pre-IND Teleconference Request
18 Nov. 2003			Pre-IND Meeting
30 Dec. 2003	IND 61,220 Serial 000		Submission of Original IND (including general information on Gaucher disease, nonclinical data, manufacturing info., and Phase I/II study - TKT025 New Protocol and IB).
19 Jan. 2004		FDA letter	FDA Correspondence: Acknowledgement of Receipt of IND and assignment of IND number
28 Jan 2004		TCR Contact Report	FDA Medical Officer request protocol amendment as discussed at the Pre-IND meeting.
28 Jan 2004	IND 61,220 Serial 001		Response to email dated 13 Jan. 2004 containing questions on clarification of age, inclusion criteria, and genotyping.
11 Mar. 2004	IND 61,220 Serial 002		Protocol Amendment: Amendment 2 of Clinical Protocol TKT025
07 April 2004	IND 61,220 Serial 003		Protocol Amendment: New Investigator for TKT025 and Blinding procedures used in TKT025.
20 May 2004		TCR Contact Report	FDA Medical Officer says it's safe to proceed with the blinding procedure as amended in Serial 003 dated 07 April 2004 for Study TKT025.
25 Aug. 2004		FDA letter	Re: Completion of IND Preclinical Pharm/Tox review and comments/recommendations
02 Nov. 2004	IND 61,220 Serial 007		Protocol Amendment: TKT025 Protocol Amendment 4 and New Protocol TKT025EXT
24 Nov. 2004	IND 61,220 Serial 008		Information Amendment: Comparability Protocol – comprehensive plan for evaluating changes to manufacturing process for drug substance (switch from 3x 30L Bioreactor to 100 L Bioreactor).
06 Apr.	IND 61,220 Serial		Information Amendment: Pharm/Tox: Final Study

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Date	IND or NDA Serial No. (if applicable)	Other Contact	Description
2005	017		Reports: Rat & Monkey 6-Month tox studies.
04 Nov. 2005	IND 61,220 Serial 023		Type B Meeting (End of Phase II) Request
12 Dec. 2005	IND 61,220 Serial 024		End of Phase II Briefing Document
09 Jan. 2006		FDA Fax	FDA responses to EOP II Meeting questions
11 Jan. 2006			End of Phase II Meeting
07 Feb. 2006		FDA letter	Official FDA Minutes from EOPII meeting 11 Jan. 2006
30 Mar. 2006	IND 61,220 Serial 029		Information Amendment: CMC: Manufacturing process modifications: switch from 3x 30L to 500 L scale up (AF1 process).
12 April 2006	IND 61,220 Serial 030		Information Amendment: Pharm/Tox: supportive information to justify Nonclinical Development Program and request for teleconference
02 May 2006	IND 61,220 Serial 031		Type A Meeting Request to discuss adequacy of nonclinical development program to initiate P3 studies and support a NDA.
18 May 2006	IND 61,220 Serial 032		Type C Meeting on 16 June 2006: Pharm/Tox Briefing Package
15 June 2006		FDA Fax	FDA Correspondence: 16 June 2006 teleconference not necessary, based on FDA'S initial responses to questions.
23 June 2006		FDA letter	FDA Correspondence: Comments and request for additional information, re: amendment dated 12 April 2006, IND Serial 030.
12 July 2006		FDA letter	FDA Correspondence: Acknowledgement of Shire's decision to accept FDA's written responses in lieu of meeting.
28 July 2006	IND 61,220 Serial 034		Information Amendment: Pharm/Tox: Responses to FDA comments and requests to 23 June fax, rat and rabbit studies.
03 Aug. 2006	IND 61,220 Serial 035		Information Amendment: CMC: Description and comparability data of AF1 process material
22 Sept. 2006	IND 61,220 Serial 037		Protocol Amendment: New Protocol , Phase 2/3 Clinical Protocol TKT032
16 Nov. 2006	IND 61,220 Serial 039		Protocol Amendment: New Protocol , Phase 3 Clinical Protocol TKT034
20 Nov. 2006		TCR Contact Report	Teleconference: IND put on clinical hold over concerns about product comparability.
28 Nov.		FDA Fax	FDA Correspondence: Full Clinical Hold Letter

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Date	IND or NDA Serial No. (if applicable)	Other Contact	Description
2006			
28 Nov. 2006	IND 61,220 Serial 040		Type A Meeting Request, response to Clinical Hold.
29 Nov. 2006		TCR Contact Report	Teleconference: response to FDA concerns relating to DS comparability resulting in Full Clinical Hold.
30 Nov. 2006	IND 61,220 Serial 041		Complete Response to Full Clinical Hold Letter relating to DS comparability.
30 Nov. 2006	IND 61,220 Serial 042		Request for Partial Waiver of the Full Clinical Hold.
01 Dec. 2006		TCR Contact Report	Teleconference: FDA agrees to move the Full Clinical Hold to a partial hold after reviewed Shire's response.
07 Dec. 2006		FDA letter	FDA Correspondence: Partial Clinical Hold Letter
12 Dec. 2006	IND 61,220 Serial 043		Response to Clinical/Statistical non-hold issues raised in full Clinical Hold Letter (Study TKT032)
14 Dec. 2006	IND 61,220 Serial 044		Protocol Amendment for Study TKT025EXT.
21 Dec. 2006		FDA letter	FDA Correspondence: Removal of Partial Clinical Hold Note: all clinical issues have been resolved.
26 Feb. 2007	IND 61,220 Serial 047		Protocol Amendment: New Protocol HGT-GCB-039 and New Investigator for TKT032
04 Sept. 2007		FDA letter	FDA Correspondence: Request for Information-Study TKT034
24 Sept. 2007	IND 61,220 Serial 055		Response to FDA request for Information-Study TKT032
10 Dec. 2007	IND 61,220 Serial 057		Response to FDA Request for Information-Study TKT034
11 Dec. 2007	IND 61,220 Serial 058		Information Amendment: Comparability Protocol for drug substance cell culture scale-up (AF2) vs. AF1 process
19 Dec 2007	IND 61,220 Serial 060		Protocol Amendment: New Protocol HGT-GCB-044 (Extension study for TKT032, TKT034 and HGT- GCB-039).
03 Sept. 2008	IND 61,220 Serial 070		Information Amendment: Description and comparability data of AF2 process material
06 Oct. 2008	IND 61,220 Serial 072		Information Amendment: Description and comparability data of 200 U/vial presentation
30 April 2009			Request for Orphan Drug Designation to FDA OOPD
08 June 2009	IND 61,220 Serial 081		Type B Meeting Request: Pre-NDA Meeting
08 June 2009		FDA OOPD	FDA OOPD Correspondence: Orphan Drug Designation Granted, US ODD #09-2835

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Date	IND or NDA Serial No. (if applicable)	Other Contact	Description
		letter	
30 June 2009	IND 61,220 Serial 082		Submission of Treatment Protocol HGT-GCB-058
30 June 2009	IND 61,220 Serial 083		Request for Fast Track Designation
01 July 2009		FDA letter	FDA Correspondence: Type B Pre-NDA meeting Granted on 10 August, 2009
08 July 2009		FDA letter	FDA Correspondence: Acknowledgement of Fast Track designation Request
15 July 2009		FDA letter	FDA Correspondence: Fast Track Designation Granted
23 July 2009		TCR Contact Report	Plans for rolling NDA for velaglucerase alfa
27 July 2009	IND 61,220 Serial 088		Request for Submission of Portions of an NDA Application
29 July 2009		WHO Collaboratin g Centre for Drug Statistics Methodology	ATC application for velaglucerase alfa (Ref: 09/1527- 2/EPLI/TUGR). ATC Proposed Code: A16AB10 velaglucerase alfa.
30 July 2009		FDA letter	FDA Correspondence: Acknowledgement to Proceed with Treatment Protocol HGT-GCB-058
30 July 2009	NDA 022575, Sequence 0000		Submission of 1st wave Rolling NDA, including M3 (complete), M4 (complete), M5 (partial). And request for Priority Review of NDA
08 Aug. 2009		FDA Fax	FDA Correspondence: FDA preliminary response for Pre- NDA Meeting Briefing Package
10 Aug. 2009			Pre-NDA Meeting
31 Aug. 2009	NDA 022575, Sequence 0001		Submission of 2nd wave of Rolling NDA, including M1, M2 (complete), M3 (update), M5 (complete).
14 Sept. 2009		FDA email, fax, letter	FDA Correspondence: FDA Acknowledgement Letter of NDA Submission
22 Sept. 2009	NDA 022575, Sequence 0003		Request for Proprietary Name Review
01 Oct. 2009	NDA 022575, Sequence 0005		Trade Name Request –Labeling Supplement
30 Oct. 2009		FDA letter	FDA Correspondence: Filing Communication – Priority Review Granted, and a list of review questions included.
03 Nov. 2009	NDA 022575, Sequence 0011		Location of data to support Orphan Drug Designation
19 Nov.		FDA Fax	Request Clinical Information

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Date	IND or NDA Serial No. (if applicable)	Other Contact	Description
2009			
20 Nov. 2009		FDA Email String	FDA Inspection Announcement Letters - 2 sites Inspections (Dec 6-10, 14-18, 2009)
20 Nov. 2009	NDA 022575, Sequence 0012		Partial Response to Request for Information: FDA Questions dated 30 Oct. 2009
01 Dec. 2009	NDA 022575, Sequence 0013		3 Month Safety Update
04 Dec. 2009		Email String	FDA BIMO Visit to Shire HGT LTP Site (Dec 08, 2009)
04 Dec. 2009	NDA 022575, Sequence 0014		Complete Response to Request for Information: FDA Questions dated 30 Oct. 2009 and 19 Nov. 2009
11 Dec. 2009		FDA Fax	Request CMC, Clinical Information
16 Dec. 2009		FDA letter	Proprietary name request : Conditional acceptance
18 Dec. 2009	NDA 022575, Sequence 0016		Response to Request for Information: FDA letter dated 11 Dec. 2009 (CMC)
22 Dec. 2009	NDA 022575, Sequence 0017		Stability update (drug substance and drug product)
31 Dec. 2009	NDA 022575, Sequence 0018		Response to Request for Information: Quality and Efficacy
13 Jan. 2010	NDA 022575, Sequence 0019		Response to Request for Information: CMC Questions of 23 Dec. 2009 and 07 Jan 2010 letters
14 Jan. 2010	NDA 022575, Sequence 0020		Response to to Request for Information: Clinical Questions of 23 Dec. 2009 Fax
15 Jan. 2010	NDA 022575, Sequence 0021		Response to telephone request of 15 Jan. 2010 – CMC information
26 Jan. 2010	NDA 022575, Sequence 0022		Response to Request for Information regarding inspections: Responses to FDA Form 483 in Paraguay, Israel, Shire HGT (300 PW)
27 Jan. 2010	NDA 022575, Sequence 0023		Response to 22 Jan. 2010 FDA Request for CMC Information
29 Jan. 2010		FDA email & letter	FDA comments on US PI
01 Feb. 2010	NDA 022575, Sequence 0024		Response to 27 Jan. 2010 FDA Request for Clinical Information
08 Feb. 2010	NDA 022575, Sequence 0025		Response to 03 Feb. 2010 FDA Fax Request for Clinical Information
09 Feb. 2010	NDA 022575, Sequence 0026		Response to FDA labeling question dated 29 Jan. 2010
10 Feb. 2010		FDA email &	FDA comments on labeling-carton labeling and container labels

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Date	IND or NDA Serial No. (if applicable)	Other Contact	Description
		letter	
17 Feb. 2010		FDA email	Response to FDA fax 10 Feb 2010 on labeling-carton label and vial labels
17 Feb. 2010	NDA 022575, Sequence 0027		Response to FDA labeling question dated 10 Feb. 2010 (carton and container comments)
18 Feb. 2010		FDA Fax	FDA comments on labeling-carton and container labels
19 Feb. 2010	NDA 022575, Sequence 0028		Response to FDA labeling comments 17 Feb 2010 and carton and container label comments 18 Feb 2010
25 Feb. 2010	NDA 022575, Sequence 0029		Information Amendment: Final Post-Marketing Commitments and Final Labeling Text (Company agreed PMCs and labeling text)
25 Feb. 2010	NDA 022575, Sequence 0030		Information Amendment: Post-Marketing Commitments and Draft Labeling Text
26 Feb. 2010		FDA Action Letter	NDA Approval Letter

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(12) Statement Concerning Eligibility for and Duration of Extension

Sought Under 35 U.S.C. § 156 [37 C.F.R. §1.740(a)(12)]

(i) Applicant is of the opinion that U.S. Patent No. 7,138,262 B1 is eligible for extension of the patent term under 35 U.S.C. § 156 of 687 days and should be extended until July 6, 2022. It satisfies all requirements for such extension including:

(a) 35 U.S.C. § 156(a) - U.S. Patent No. 7,138,262 B1 claims a method of manufacturing the approved product, VPRIV™.

(b) 35 U.S.C. § 156(a)(1) - U.S. Patent No. 7,138,262 B1 has not expired before submission of this application.

(c) 35 U.S.C. § 156(a)(2) - The term of U.S. Patent No. 7,138,262 B1 has never been extended under 35 U.S.C. § 156(e)(1).

(d) 35 U.S.C. § 156(a)(3) - The application for patent term extension is submitted by the owner of record of the patent in accordance with the requirements of paragraphs (1) through (4) of 35 U.S.C. § 156(d) and the rules of the Patent and Trademark Office.

(e) 35 U.S.C. § 156(a)(4) - The product VPRIV™ has been subject to a regulatory review period before its commercial marketing or use.

(f) 35 U.S.C. § 156(a)(5)(A) - The commercial marketing or use of the product VPRIV™ after the regulatory review period is the first permitted commercial marketing or use under the provisions of § 505(b) of the Federal Food, Drug, and Cosmetic Act under which such regulatory review period occurred.

(g) 35 U.S.C. § 156(c)(4) - No other patent has been extended for the same regulatory review period for the product VPRIV™.

(h) This application is being submitted within 60 days of regulatory agency approval.

(i) This application otherwise complies with all requirements of 35 U.S.C. § 156 and all applicable rules and procedures.

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(12)(ii) Applicant respectfully submits that the length of the extension of patent term for U.S. Patent No. 7,138,262 B1 is 687 days pursuant to 35 U.S.C. § 156(c).

The length of the extension was determined pursuant to 37 C.F.R. § 1.775 as follows (the remainder of this section (12)(ii) is numbered so as to correspond to the numbering in 37 C.F.R. § 1.775):

(c) The regulatory review period under 35 U.S.C. § 156(g)(1)(B) is a total of 2,110 days, which is the sum of (1) and (2) below:

(1) The period of review under 35 U.S.C. § 156(g)(1)(B)(i), which is the number of days in the period beginning on the date the exemption became effective (May 20, 2004) and ending on the date an application was initially submitted (August 31, 2009), which is 1,930 days; and

(2) The period of review under 35 U.S.C. § 156(g)(1)(B)(ii), which is the number of days in the period beginning on the date the application was initially submitted (August 31, 2009) and ending on the date such application was approved (February 26, 2010), which is 180 days.

(d) The term of the patent as extended for a human drug, antibiotic drug or human biological product is determined by:

(1) Subtracting from the number of days determined to be in the regulatory review period, which is 2,110:

(i) The number of days in the regulatory review period which were on or before the date on which the patent issued (November 21, 2006) which is 916 days; and

(ii) The number of days in the period of (c)(1) and (c)(2) above during which applicant did not act with due diligence, which is zero (0) days; and

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

(iii) One-half the number of days determined in subparagraph (c)(1) above after that period is reduced by subparagraph (d)(1)(i) and (d)(1)(ii) which, is $(1,930-916-0)/2$, or 507 days.

Thus, the number of days determined in subparagraph (c) above (2,110) is reduced by 1,423 $(916+507)$ days, for a total of 687 days;

(2) Adding the number of days as determined in subparagraph (d)(1), (687 days), to the original term of the patent (August 18, 2020) which results in the date of July 6, 2022.

(3) By adding fourteen (14) years to the date of approval of the New Drug Application (NDA 22575) which results in the date of February 26, 2024;

(4) By comparing the dates for the ends of the periods obtained pursuant to paragraphs (d)(2) and (d)(3) and selecting the earlier, which is July 6, 2022;

(5) (i) Since U.S. Patent No. 7,138,262 B1 issued after September 24, 1984, by adding 5 years to the original expiration date of the patent or any earlier date set by terminal disclaimer, which is August 18, 2025; and (ii) By comparing the dates obtained pursuant to paragraphs (d)(4) and (d)(5)(i) of this section with each other and selecting the earlier date, which is July 6, 2022.

Thus, the patent is entitled to extension until July 6, 2022.

(13) Statement Pursuant to 37 C.F.R. § 1.740(a)(13)

Applicant acknowledges a duty to disclose to the Director of the United States Patent and Trademark Office and the Secretary of Health and Human Services any information which is material to the determination of entitlement to the extension sought, e.g., as that duty is defined in 37 C.F.R. § 1.765.

Issued: November 21, 2006

Inventors: Peter Francis Daniel

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Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
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(14) Applicable Fee [1.740(a)(14)]

The prescribed fee for receiving and acting upon this application is attached as a check in the amount of \$1,120.00. The Director is authorized to charge any additional fees required by this application to Deposit Account No. 50/2762, referencing attorney docket number S2071-701019.


(15) Name and Address for Correspondence [1.740(a)(15)]

All correspondence and inquiries may be directed to the undersigned, whose address, telephone number and fax number are as follows:

Laurie Butler Lawrence
Lando & Anastasi, LLP
One Main Street
Cambridge, MA 02142
Phone: 617-395-7000
Fax: 617-395-7070

Enclosed is a certification that the application for extension of patent term under 35 U.S.C. § 156 including its attachments and supporting papers is being submitted as one original and two (2) copies thereof (Attachment K) in compliance with 37 C.F.R. § 1.740(b).

Respectfully submitted,

By: 
Laurie Butler Lawrence, Reg. No. 46,593
LANDO & ANASTASI, LLP
One Main Street
Cambridge, Massachusetts 02142
United States of America
Telephone: 617-395-7000
Facsimile: 617-395-7070

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Date: April 22, 2010

Attachments:

Power of Attorney (Attachment A)

Package Insert for VPRIVTM (Attachment B)

NDA approval letter from FDA to Shire Human Genetic Therapies, Inc., dated
February 26, 2010 (with enclosure) (Attachment C)

U.S. Patent No. 7,138,262 B1 (Attachment D)

Maintenance Fee Statement (Attachment E)

Brumshtein et al. (2010) Glycobiology 20(1):24-32 (Attachment F)

Letter from FDA to Transkaryotic Therapies, Inc., dated January 12, 2004,
providing the IND number and showing the date of receipt by FDA of the IND
(Attachment G)

A written record of the discussion that occurred on January 28, 2004 regarding
modification of the protocol (Attachment G1)

Letter from Transkaryotic Therapies, Inc. to FDA dated March 11, 2004,
concerning amendment of protocol (Attachment G2)

FDA communication to Transkaryotic Therapies, Inc., dated May 20, 2004,
concerning amendment to protocol (Appendix G3).

Letter from FDA to Shire Human Genetics Therapies, Inc. indicating the date the
IND was put on clinical hold (Attachment H)

Letter from FDA to Shire Human Genetics Therapies, Inc., dated December 21,
2006, removing the clinical hold and indicating that the protocol can be initiated
(Attachment I)

Letter from FDA to Shire Human Genetics Therapies, Inc., dated September 14,
2009, acknowledging receipt of the final submission of the NDA (Attachment J)

Certification of Copies of Application Papers (Attachment K)

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

**Assignee: Shire Human Genetic Therapies,
Inc.**

**Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS**

Attachment A

Power of Attorney

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REVOCATION OF PRIOR POWERS OF ATTORNEY
and
NEW POWER OF ATTORNEY

Sir:

The undersigned, Shire Human Genetic Therapies, Inc., a Delaware Corporation, assignee of the entire right, title and interest for all of the patents and patent applications identified in the attached Schedule A, hereby revokes all previous powers of attorney or authorizations of agent given in the identified patents and patent applications and in any divisional, continuing, substitute, renewal, reexamination, or reissue applications thereof, and appoints all practitioners of Lowrie, Lando & Anastasi, LLP associated with Customer Number:

37462

as assignee's attorneys or agents with full power of substitution to take any and all action necessary with regard to the identified patents and patent applications, and with regard to any divisional, continuing, substitute, renewal or reissue applications thereof.

Please address all telephone calls to Laurie Butler Lawrence at telephone no. (617) 395-7000.

Please forward all correspondence to the correspondence address associated with

Customer Number:

37462

Shire Human Genetic Therapies, Inc.

By:

Name: Kerry A. Flynn

Title: Vice President, Intellectual Property

Dated:

April 14, 2008

ASSIGNEE CERTIFICATION

Attached to this power is a Certificate Under 37 CFR 3.73(b).

Dated:

April 17, 2008

Natalie A. Lissy
Natalie A. Lissy, Reg. No. 59,651
LOWRIE, LANDO & ANASTASI, LLP
Riverfront Office Park
One Main Street
Cambridge, MA 02142
(617) 395-7000

SCHEDULE A

U.S. Patents:

<u>U.S. PATENT NO.</u>	<u>ISSUE DATE</u>	<u>ATTORNEY'S DOCKET NO.</u>
6,924,365	08/02/2005	S2071-700410
7,229,793	06/12/2007	S2071-700719
6,569,681	05/27/2003	S2071-700919
7,138,262	11/21/2006	S2071-701019
5,965,125	10/12/1999	S2071-701419
6,472,181	10/29/2002	S2071-701440
6,582,391	06/24/2003	S2071-701441
6,083,725	07/04/2000	S2071-701510
6,566,099	05/20/2003	S2071-701520
7,122,354	10/17/2006	S2071-701521
6,395,884	05/28/2002	S2071-701540
5,817,789	10/06/1998	S2071-701619
6,027,921	02/22/2000	S2071-701640
6,262,026	07/17/2001	S2071-701641
6,858,578	02/22/2005	S2071-701642
6,419,920	07/16/2002	S2071-701730
6,458,574	10/01/2002	S2071-702030

SCHEDULE A

U.S. Patent Applications:

<u>U.S. APPLICATION NO.</u>	<u>FILING DATE</u>	<u>ATTORNEY'S DOCKET NO.</u>
11/581,979	10/17/2006	S2071-701040
11/028,850	01/03/2005	S2071-701620
10/160,452	05/31/2002	S2071-701740
10/165,060	07/07/2002	S2071-702040
11/403,618	04/13/2006	S2071-702540
11/671,588	02/06/2007	S2071-702719
10/775,678	02/10/2004	S2071-702810
08/712,614	09/13/1996	S2071-703119
10/423,225	04/25/2003	S2071-702510
09/686,497	10/11/2000	S2071-701319
11/924,804	10/26/2007	S2071-701320
11/925,125	10/26/2007	S2071-701321
11/925,167	10/26/2007	S2071-701322
11/928,247	10/30/2007	S2071-701323
10/165,968	06/10/2002	S2071-702020
60/375,584	04/25/2002	S2071-702500
60/771,555	02/07/2006	S2071-702700
10/968,870	10/18/2004	S2071-701020

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

STATEMENT UNDER 37 CFR 3.73(b)Applicant/Patent Owner: Peter Francis Daniel et al.Application No./Patent No.: 7,138,262 Filed/Issue Date: 11/21/2006Entitled: HIGH MANNOSIDE PROTEINS AND METHODS OF MAKING HIGH MANNOSIDE PROTEINSShire Human Genetics Therapies, Inc., a Delaware Corporation
(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that it is:

1. ☒ the assignee of the entire right, title, and interest; or
2. ☐ an assignee of less than the entire right, title and interest
(The extent (by percentage) of its ownership interest is _____ %)

in the patent application/patent identified above by virtue of either:

A ☐ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel _____, Frame _____, or for which a copy thereof is attached.

OR

B ☒ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: Peter Francis Daniel et al. To: Transkaryotic Therapies, Inc.
The document was recorded in the United States Patent and Trademark Office at
Reel 011662, Frame 0815, or for which a copy thereof is attached.
2. From: Transkaryotic Therapies, Inc. To: Shire Human Genetics Therapies, Inc.
The document was recorded in the United States Patent and Trademark Office at
Reel 018224, Frame 0390, or for which a copy thereof is attached.
3. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at
Reel _____, Frame _____, or for which a copy thereof is attached.

☐ Additional documents in the chain of title are listed on a supplemental sheet.

☒ As required by 37 CFR 3.73(b)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (i.e., a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

/Natalie A. Lissy/April 24, 2008

Signature

Date

Natalie A. Lissy, Reg. No. 59,651617-395-7000

Printed or Typed Name

Telephone Number

Attorney

Title

This collection of information is required by 37 CFR 3.73(b). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

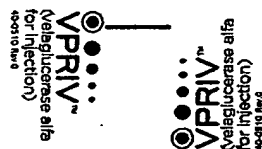
Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment B

Package Insert for VPRIV™



HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use VPRIV safely and effectively. See full prescribing information for VPRIV.

VPRIV (valganciclovir) for injection
Initial U.S. Approval: 2010

INDICATIONS AND USAGE

VPRIV (valganciclovir) for injection is a hydroxy (prodrug) phosphonamide-specific enzyme indicated for long-term suppressive therapy (ERT) for pediatric and adult patients with type 1 Cerebral disease (1).

DOSE AND ADMINISTRATION

- 60 Units/kg administered every other week as a 60-minute intravenous infusion (2).
- Patients currently being treated with valganciclovir for Cerebral disease can be switched to VPRIV. Patients previously treated on a stable dose of valganciclovir are recommended to begin treatment with VPRIV at that same dose when they switch from valganciclovir to VPRIV (2).
- Physicians can make dosage adjustments based on achievement and maintenance of each patient's therapeutic goals. Clinical trials have evaluated doses ranging from 15 Units/kg to 60 Units/kg every other week (2).

DOSE FORMS AND STRENGTHS

- Lyophilized powder to be reconstituted and diluted for infusion (3).
- Available in 200 Units and 400 Units single-use vials (3).

CONTRAINDICATIONS

- None (4).

WARNINGS AND PRECAUTIONS

- Hypersensitivity reactions: Treatment with VPRIV should be carefully re-evaluated in the presence of significant evidence of hypersensitivity to the product (5.1).
- Infection-related reactions (5.2).

ADVERSE REACTIONS

- The most common adverse reactions during clinical studies were infection-related reactions (5.2, 6.1).
 - Other commonly observed adverse reactions in a 10% of patients were: headache, diarrhea, abdominal pain, nausea, back pain, joint pain, upper respiratory tract infection, activated PTI, pharyngitis, lymphadenitis, and pyrexia (6.2).
- To report SUSPECTED ADVERSE REACTIONS, contact Shire Human Genetic Therapies, Inc. at the OnePactSM phone 1-866-833-0680 or MedWatch@shire.com, or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch. See Section 17 for PATIENT COUNSELING INFORMATION.

Revised: 02/2016

FULL PRESCRIBING INFORMATION: CONTENTS

1. INDICATIONS AND USAGE
2. DOSAGE AND ADMINISTRATION
 - 2.1 Recommended Dose
 - 2.2 Preparation and Administration Instructions
3. DOSAGE FORMS AND STRENGTHS
4. CONTRAINDICATIONS
5. WARNINGS AND PRECAUTIONS
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 - 5.2 Infection-related Reactions
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10. OVERDOSAGE
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12. CLINICAL PHARMACOLOGY
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16. HOW SUPPLIED/STORAGE AND HANDLING
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17. PATIENT COUNSELING INFORMATION

*Details on contraindications, warnings, and precautions are not listed.

VPRIVTM (valganciclovir) for injection

FULL PRESCRIBING INFORMATION

1. INDICATIONS AND USAGE

VPRIV (valganciclovir) for injection is a hydroxy (prodrug) phosphonamide-specific enzyme indicated for long-term suppressive therapy (ERT) for pediatric and adult patients with type 1 Cerebral disease.

2. DOSAGE AND ADMINISTRATION

2.1 Recommended Dose

The recommended dose is 60 Units/kg administered every other week as a 60-minute intravenous infusion. Patients currently being treated with valganciclovir for type 1 Cerebral disease may be switched to VPRIV. Patients previously treated on a stable dose of valganciclovir are recommended to begin treatment with VPRIV at that same dose when they switch from valganciclovir to VPRIV.

Dose adjustments can be made based on achievement and maintenance of each patient's therapeutic goals. Clinical studies have evaluated doses ranging from 15 Units/kg to 60 Units/kg every other week. VPRIV should be administered under the supervision of a healthcare professional.

2.2 Preparation and Administration Instructions

Use aseptic technique

VPRIV is a lyophilized powder, which requires reconstitution and dilution, and is intended for intravenous infusion only. VPRIV contains no preservatives and should be single-use only. Clinical use may require reconstitution. VPRIV should be prepared as follows:

Determine the number of vials to be reconstituted based on the individual patient's weight and the prescribed dose. Follow the instructions in Table 1 for reconstitution.

Table 1: Reconstitution Instructions

	200 Units/vial	400 Units/vial
Volumes of Sterile Water for Injection, USP for reconstitution	2.2 mL	4.3 mL
Concentration after reconstitution	100 Units/mL	100 Units/mL
Withdrawal volume	2 mL	4 mL

Upon reconstitution, vials will contain 60 Units/kg. Prior to further dilution, visually inspect the solution in the vials; the solution should be clear to slightly opalescent and colorless. Do not use if the solution is discolored or if foreign particulate matter is present. Withdraw the calculated volume of drug from the appropriate number of vials and dilute the total volume required in 100 mL of 0.9% sodium chloride solution suitable for IV administration. Mix gently. DO NOT SHAKE.

VPRIV should be administered over 60 minutes. VPRIV should not be mixed with other products in the same infusion tubing as the compatibility in solution with other products has not been evaluated. The diluted solution should be stored through an in-line low protein-binding 0.2 µm filter during administration.

As VPRIV contains no preservatives, once reconstituted the product should be used immediately. If immediate use is not possible, the reconstituted or diluted product may be stored for up to 24 hours at 2 to 8°C (36 to 46°F). Do not freeze. Protect from light. The solution should be completed within 24 hours of reconstitution of vials.

3. DOSAGE FORMS AND STRENGTHS

VPRIV is a sterile, white to off-white, lyophilized powder for reconstitution with Sterile Water for Injection, USP, to yield a final concentration of 100 Units/mL.

VPRIV is available in 200 Units and 400 Units single-use vials.

4. CONTRAINDICATIONS

None.

5. WARNINGS AND PRECAUTIONS

5.1 Hypersensitivity Reactions

Hypersensitivity reactions have been reported in patients in clinical studies with VPRIV (see Adverse Reactions (6.2)). As with any intravenous product, hypersensitivity reactions are possible. Therefore appropriate medical support should be readily available when VPRIV is administered. If a severe reaction occurs, correct medical standards for emergency treatment are to be followed.

Treatment with VPRIV should be approached with caution in patients who have exhibited symptoms of hypersensitivity to the active ingredient or components in the drug product or to other suppressive therapy.

5.2 Infection-related Reactions

Infection-related reactions were the most commonly observed adverse reactions in patients treated with VPRIV in clinical studies. The most commonly observed infection-related reactions were headache, diarrhea, upper respiratory tract infection, nausea, pharyngitis, and pyrexia. Generally, the infection-related reactions were mild and, in treatment-naïve patients, onset occurred during the first 6 months of treatment and tended to occur less frequently with time.

The management of infection-related reactions should be based on the severity of the reaction, e.g., during the infusion rate, treatment with medications such as antidiarrheals, antipyretics and/or corticosteroids, and/or stopping and restarting treatment with increased infusion time.

Pre-treatment with antihistamines and/or corticosteroids may prevent subsequent reactions in those cases where appropriate treatment was required. Patients were not routinely pre-medicated prior to infusion of VPRIV during clinical studies.

6. ADVERSE REACTIONS

6.1 Clinical Studies Experience

The data described below reflect exposure of 94 patients with type 1 Cerebral disease who received VPRIV at doses ranging from 15 Units/kg to 60 Units/kg every other week in 5 clinical studies. Fifty-four (58%) patients were naive to ERT and received VPRIV for 9 months and 40 patients received from valganciclovir to VPRIV treatment and received VPRIV for 12 months (see Clinical Studies (14.2)). Patients were between 4 and 21 years old at time of first treatment with VPRIV, and included 46 males and 48 females.

The most common adverse reactions in patients treated with VPRIV were hypersensitivity reactions (see Warnings and Precautions (5.1)).

The most commonly reported adverse reactions (occurring in ≥10% of patients) that were considered related to VPRIV are shown in Table 2. The most common adverse reactions were infection-related reactions.

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice.

Table 2: Adverse Reactions Observed in a 10% of Patients with Type 1 Cerebral Disease Treated with VPRIV

System Organ Class Preferred Term	Naïve to ERT N = 54	Switched from valganciclovir to VPRIV N = 40
	Number of Patients (%)	
Nervous system disorders		
Headache	19 (35.2)	12 (30)
Dizziness	12 (22.2)	3 (7.5)
Gastrointestinal disorders		
Abdominal pain	10 (18.5)	6 (15)
Nausea	3 (5.6)	4 (10)
Musculoskeletal and connective tissue disorders		
Back pain	9 (16.7)	7 (17.5)
Joint pain (arthralgia)	6 (11.1)	3 (7.5)
Infections and infestations		
Upper respiratory tract infection	17 (31.5)	12 (30)
Respiratory infections		
Activated partial thromboplastin time prolonged	6 (11.1)	2 (5)
General disorders and administration site reactions		
Infection-related reaction*	28 (51.9)	9 (22.5)
Pyrexia	12 (22.2)	5 (12.5)
Arthralgia/fatigue	7 (13)	5 (12.5)

*Dermatologic events considered related to and occurring within up to 24 hours of VPRIV infusion.

Less common adverse reactions affecting more than one patient (≥2% in the treatment-naïve group and ≥5% in patients switched from valganciclovir to VPRIV treatment) were back pain, arthralgia, rash, urticaria, flushing, hyperkalemia, and hypokalemia.

Post-marketing Patients

All adult adverse reactions to VPRIV are considered related to patients (ages 4 to 17 years). Adverse reactions were commonly seen in pediatric patients compared to adult patients include (≥10% difference) upper respiratory tract infection, rash, PTI prolonged, and pyrexia.

Immunogenicity

As with all therapeutic proteins, there is a potential for immunogenicity. In clinical studies, 1 of 54 treatment-naïve patients treated with VPRIV developed IgG class antibodies to VPRIV. In this patient, the antibodies were determined to be non-neutralizing in an in vitro assay. The infection-related reactions were reported for this patient. It is unknown if the presence of IgG antibodies to VPRIV is associated with a higher risk of infection reactions. Patients with an immune response to other organ replacement therapies who are switching to VPRIV should continue to be monitored for antibodies.

Immunogenicity assay results are highly dependent on the sensitivity and specificity of the assay. Additionally, the observed incidence of antibody positivity in an assay may be influenced by several factors, including assay methodology, sample handling, timing of sample collection, concomitant medications, and underlying disease. For these reasons, comparison of the incidence of antibodies to VPRIV with the incidence of antibodies to other products may be misleading.

7. DRUG INTERACTIONS

No drug-drug interaction studies have been conducted.

8. USE IN SPECIFIC POPULATIONS

8.1 Pregnancy—Category B

Reproductive studies with valganciclovir have been performed in pregnant rats at intravenous doses up to 17 mg/kg/day (102 mg/kg/day, about 1.8 times the recommended human dose of 60 Units/kg/day or 13.5 mg/kg/day based on the body surface area). Reproductive studies have been performed in pregnant rabbits at intravenous doses up to 30 mg/kg/day (240 mg/kg/day, about 4.3 times the recommended human dose of 60 Units/kg/day based on the body surface area). These studies did not reveal any evidence of impaired fertility or harm to the fetus due to valganciclovir. A pre- and postnatal development study in rats showed no evidence of any adverse effect on pre- and postnatal development at doses up to 17 mg/kg/day (102 mg/kg/day, about 1.8 times the recommended human dose of 60 Units/kg/day based on the body surface area). There are, however, no adequate and well-controlled studies in pregnant women. Because animal reproduction studies are not always predictive of human response, VPRIV should be used during pregnancy only if clearly needed.

8.2 Nursing Mothers

There are no data from studies in lactating women. It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when VPRIV is administered to a nursing woman.

8.4 Pediatric Use

The safety and effectiveness of VPRV have been established in patients between 4 and 17 years of age. Use of VPRV in this age group is supported by evidence from adequate and well-controlled studies of VPRV in adults and pediatric (20 of 94 [21%]) patients. The safety and efficacy profiles were similar between pediatric and adult patients (see Adverse Effects (5.3) and Clinical Studies (14)). The safety of VPRV has not been established in pediatric patients younger than 4 years of age.

8.5 Geriatric Use

During clinical studies 4 patients aged 65 or older were treated with VPRV. Clinical studies of VPRV did not include sufficient numbers of subjects aged 65 and over to determine whether they respond differently than younger subjects. Other reported clinical experience has not identified differences in response between the elderly and younger patients. In general, dose reduction for an elderly patient should be approached cautiously, considering potential renal/hepatic conditions.

10 OVERDOSAGE

There is no experience with overdose of VPRV.

11 DESCRIPTION

The active ingredient of VPRV is vedolizumab, which is produced by gene activation technology in a human B-lymphocyte cell line. Vedolizumab is a glycoprotein of 497 amino acids with a molecular weight of approximately 63 kDa. Vedolizumab is a human IgG1 antibody with a heavy chain constant region of 3 domains and a light chain constant region of 2 domains. Vedolizumab is manufactured to contain predominantly heavy chain constant region 3 domains. The high mannose type N-linked glycan chains are specifically recognized and internalized via the mannose receptor present on the surface of macrophages, the cells that accumulate phagocytosis in Crohn's disease. Vedolizumab also targets the hydrolysis of the glycosylated glucocorticoids to glucose and corticoids in the lysosomes.

VPRV is stored by Unilever, where one Unit of activity is defined as the quantity of enzyme required to convert one micromole of p-nitrophenyl 4-O-glucopyranoside to p-nitrophenol per minute at 37°C.

VPRV is supplied as a sterile, preservative free, lyophilized powder in single-use vials. Following reconstitution with Sterile Water for Injection, USP, the solution contains the components listed in Table 3.

Table 3: VPRV Composition Following Reconstitution

	Extractable 200 Units/vial	Extractable 400 Units/vial
Active ingredient		
vedolizumab	200 Units	400 Units
Inactive ingredients		
citric acid, anhydrous	2.52 mg	5.04 mg
polysorbate 20	0.22 mg	0.44 mg
sodium citrate, dihydrate	25.68 mg	51.36 mg
sucrose	900 mg	200 mg

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

Crohn's disease is an intestinal mucosal disorder caused by mutations in the CARD15 gene, which results in a deficiency of the lysosomal enzyme beta-glucuronidase. Glucuronidase catalyzes the conversion of the sphingolipid glucosaminidase into glucose and ceramide. The enzymatic deficiency causes an accumulation of glucosaminidase primarily in the lysosomes of macrophages, giving rise to foam cells or "foamy cells." In this lysosomal storage disorder (LSD), clinical features are reflective of the accumulation of glucosaminidase in the liver, spleen, bone marrow, and other organs. The accumulation of glucosaminidase in the liver and spleen leads to hepatomegaly. Presence of glucosaminidase in the bone marrow and spleen lead to clinically significant anemia and thrombocytopenia.

Vedolizumab also targets the hydrolysis of glucosaminidase, reducing the amount of accumulated glucosaminidase.

12.3 Pharmacokinetics

In a multicenter study conducted in patients (16-7, 4 to 17 years old) and adult (18-55, 19 to 62 years old) patients with type 1 Crohn's disease, pharmacokinetic parameters were performed at Weeks 1 and 37 following 60-minute intravenous infusions of VPRV 60 Unit/kg every other week. Serum vedolizumab concentrations declined rapidly with a mean half-life of 11 to 12 minutes. The mean vedolizumab half-life ranged from 6.72 to 7.56 minutes. The mean volume of distribution at steady state ranged from 82 to 100 L (16.2% to 10.7% of body weight). However, because an inadequately validated analytical assay method was used in the calculations, the accuracy and reliability of pharmacokinetic parameter values are not currently available. No accumulation or change in vedolizumab pharmacokinetics over time from Weeks 1 to 37 was observed upon multiple-dosing 60 Unit/kg every other week. Based on the limited data, there were no notable pharmacokinetic differences between male and female patients in this study. The effect of age on pharmacokinetics of vedolizumab was inconclusive.

The effect of anti-drug antibody formation on the pharmacokinetic parameters of vedolizumab was inconclusive.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenicity, Mutagenicity, Impairment of Fertility

Long-term studies in animals to evaluate carcinogenic potential or studies to evaluate mutagenic potential have not been performed with vedolizumab.

In a male and female fertility study in rats, vedolizumab did not cause any significant adverse effect on male or female fertility parameters up to a maximum dose of 17 mg/kg/day (102 mg/kg/day, about 1.8 times the recommended human dose of 60 Unit/kg/day based on the body surface area).

14 CLINICAL STUDIES

The efficacy of VPRV was assessed in three clinical studies in a total of 99 patients with type 1 Crohn's disease: 82 patients age 4 years and older received VPRV and 17 patients age 3 years and older received placebo. Studies 1 and 2 were conducted in patients who were not currently receiving Crohn's disease-specific therapy. Study 3 was conducted in patients who were receiving Crohn's disease-specific therapy before starting VPRV. In these studies, VPRV was administered intravenously every other week at doses ranging from 15 Unit/kg to 60 Unit/kg every other week.

14.1 Studies of VPRV as Initial Therapy

Study 1 was a 12-month, randomized, double-blind, active-controlled (placebo), parallel-group, multicenter study in 25 patients age 4 years and older with Crohn's disease. Patients were not allowed to have had Crohn's disease-specific therapy for at least the previous 30 months; all but one had no prior therapy. The mean age was 25 years and 60% were male. Patients were randomized to receive VPRV at a dose of either 45 Unit/kg or 60 Unit/kg (N=12) every other week.

At baseline, mean hemoglobin concentration was 12.6 g/dL, mean platelet count was 271 x 10⁹/L, mean liver values were 3.6% of body weight (ALT), and mean spleen volume was 2.3% of body weight (SPL). For all studies, liver and spleen values were measured by MRI. The changes in clinical parameters after 12 months of treatment are shown in Table 4. The observed change from baseline in the primary endpoint, hemoglobin concentration, was considered to be clinically meaningful in light of the overall history of untreated Crohn's disease.

Table 4: Mean Change from Baseline to Month 12 for Clinical Parameters in Patients with Type 1 Crohn's Disease Initiating Therapy with VPRV in Study 1

Clinical Parameter	Mean Changes from Baseline ± Std. Err. of the Mean	
	VPRV Dose (given every other week)	
	45 Unit/kg N = 13	60 Unit/kg N = 12
Hemoglobin concentration change (g/dL)	2.4 ± 0.4*	2.4 ± 0.3*
Platelet count change (x 10 ⁹ /L)	41 ± 14*	51 ± 12*
Liver values change (% BW)	-0.30 ± 0.29	-0.84 ± 0.33
Spleen volume change (% BW)	-1.9 ± 0.6*	-1.9 ± 0.5*

* Primary study endpoint was hemoglobin concentration change in the 60 Unit/kg group, p < 0.001

* Statistically significant changes from baseline after adjusting for post-baseline multiple tests

Study 2 was a 6-month, randomized, double-blind, active-controlled (placebo), parallel-group, multicenter study in 34 patients age 3 years and older. Patients were required to have Crohn's disease-related anemia and either immunosuppressive or immunomodulatory therapy for at least the previous 12 months. The mean age was 30 years and 53% were female; the youngest patient who received VPRV was age 4 years. Patients were randomized to receive either 60 Unit/kg of VPRV (N=17) or 60 Unit/kg of placebo (N=17) every other week.

At baseline, the mean hemoglobin concentration was 11.2 g/dL, mean platelet count was 171 x 10⁹/L, and mean liver values were 4.3% of body weight (ALT). For the patients who had not had splenectomy (7 in each group) the mean spleen volume was 2.4% of body weight (SPL). After 6 months of treatment, the mean absolute increase from baseline in hemoglobin concentration was 1.6 g/dL (± 0.2 SD) for patients treated with VPRV. The mean treatment difference in change from baseline to 6 months (VPRV - placebo) was 0.1 g/dL (± 0.4 SD). In Studies 1 and 2, a combination of age and gender subgroup did not identify differences in response to VPRV among these subgroups. The number of non-Crohn's patients in these studies was too small to adequately assess any differences in effects by race.

14.2 Study in Patients Switching from Injections to VPRV

Study 3 was a 12-month, open-label, single-arm, multicenter study in 40 patients age 3 years and older who had been receiving treatment with injections at doses ranging between 15 Unit/kg to 60 Unit/kg for a minimum of 10 consecutive months. Patients also were required to have a stable baseline dose of injections for at least 6 months prior to enrollment. The mean age was 30 years and 55% were female; Crohn's disease therapy was stopped, and treatment with VPRV was administered every other week at the same number of units as the patient's previous injection dose. Adjustment of doses was allowed by study cohorts if needed in order to maintain clinical parameters.

Hemoglobin concentrations and platelet counts remained stable on average through 12 months of VPRV treatment. After 12 months of treatment with VPRV the mean hemoglobin concentration was 12.5 g/dL (range: 10.8, 16.1) vs. the baseline value of 13.1 g/dL (range: 10.4, 16.3), and the mean platelet count after 12 months was 174 x 10⁹/L (range: 24, 408) vs. the baseline value of 162 x 10⁹/L (range: 25, 358). No patient received change adjustment during the 12-month treatment period.

15 REFERENCES

1. Pathways GH, Whitlock RL, Averbach R, et al. Therapeutic Goals in the Treatment of Crohn's Disease. *Smalls Research* 2004; 4(1) Suppl: S24-S34.

16 HOW SUPPLIED/STORAGE AND HANDLING

VPRV is a sterile, preservative free, lyophilized powder requiring reconstitution and further dilution prior to use. It is supplied in individually packaged glass vials, which are closed with a butyl rubber stopper with a fluorocarbon coating and are sealed with an aluminum crimped cap with a flip-off plastic cap. The vials are intended for single use only. VPRV is available as: 200 Unit/vial NDC: 540020-101-02 and 400 Unit/vial NDC: 540020-101-04.

16.1 Storage

VPRV should be stored in a refrigerator at 2 to 8°C (36 to 46°F). Do not use VPRV after the expiration date on the vial.

Do not freeze.

Protect from light.

17 PATIENT COUNSELING INFORMATION

VPRV should be administered under the supervision of a healthcare professional. VPRV is a treatment that is given intravenously (by IV) every other week. The infusion typically takes up to 60 minutes.

Patients should be advised that VPRV may cause hypersensitivity reactions or infusion-related reactions. Infusion-related reactions can usually be managed by slowing the infusion rate, treatment with medications such as antihistamines, antipyretics, and/or corticosteroids, and/or stopping and resuming treatment with increased infusion time. Prevention with antihistamines and/or corticosteroids may prevent subsequent reactions. Treatment with VPRV should be carefully re-evaluated if significant evidence of hypersensitivity in the product occurs (see Warnings and Precautions (5.1, 5.2)).

By Only

VPRV is manufactured by:

Shire Human Genetic Therapies, Inc.
700 Main Street
Cambridge, MA 02139

OneVib is a service mark and VPRV is a trademark of Shire Human Genetic Therapies, Inc.

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

**Assignee: Shire Human Genetic Therapies,
Inc.**

**Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS**

Attachment C

**NDA approval letter from FDA to Shire Human Genetic Therapies, Inc., dated
February 26, 2010 (with enclosure)**



DEPARTMENT OF HEALTH AND HUMAN SERVICES

Food and Drug Administration
Silver Spring MD 20993

NDA 022575

NDA APPROVAL

Shire Human Genetic Therapies, Inc.
Attention: Nikhil Mehta, Ph.D.
Vice President, Global Regulatory Affairs
700 Main Street
Cambridge, MA 02139

Dear Dr. Mehta:

Please refer to your new drug application (NDA) dated August 31, 2009, received August 31, 2009, submitted under section 505(b) of the Federal Food, Drug, and Cosmetic Act for VPRIV (velaglucerase alfa for injection).

We acknowledge receipt of your submissions dated July 30, August 31, September 17, 22, and 28, October 1, 9, 12, 23, and 29, November 16 and 20, December 1, 4, 15, 18, 22, and 31, 2009, and January 13, 14, 15, 26, and 27, and February 1, 8, 9, 17, 19, and 25, 2010.

This new drug application provides for the use of VPRIV (velaglucerase alfa for injection) for long-term enzyme replacement therapy (ERT) for pediatric and adult patients with type 1 Gaucher disease.

We have completed our review of this application, as amended. It is approved, effective on the date of this letter, for use as recommended in the enclosed agreed-upon labeling text.

Your application was not referred to an advisory committee because this drug is not the first in its class, the clinical study design was acceptable, the application did not raise significant safety or efficacy issues, the application did not raise significant public health questions on the role of the drug in the diagnosis, cure, mitigation, treatment or prevention of a disease, and outside expertise was not necessary.

CONTENT OF LABELING

As soon as possible, but no later than 14 days from the date of this letter, please submit the content of labeling [21 CFR 314.50(I)] in structured product labeling (SPL) format, as described at <http://www.fda.gov/ForIndustry/DataStandards/StructuredProductLabeling/default.htm>, that is identical to the submitted labeling (package insert submitted February 25, 2010). For administrative purposes, please designate this submission, "SPL for approved NDA 022575."

CARTON AND IMMEDIATE CONTAINER LABELS

We acknowledge your February 19, 2010, submission containing final printed carton and container labels.

Marketing the product with final printed labeling that is not identical to the approved labeling text may render the product misbranded and an unapproved new drug.

REQUIRED PEDIATRIC ASSESSMENTS

Under the Pediatric Research Equity Act (PREA) (21 U.S.C. 355c), all applications for new active ingredients, new indications, new dosage forms, new dosing regimens, or new routes of administration are required to contain an assessment of the safety and effectiveness of the product for the claimed indication in pediatric patients unless this requirement is waived, deferred, or inapplicable.

Because this drug product for this indication has an orphan drug designation, you are exempt from this requirement.

POSTMARKETING COMMITMENTS SUBJECT TO REPORTING REQUIREMENTS UNDER SECTION 506B

We remind you of your postmarketing commitments in your submission dated February 25, 2010. These commitments are listed below.

- 1600-01 Shire commits to utilize an antibody screening cut point based on a mean + 1.645 standard deviation for assay values from treatment naïve Gaucher patients. Shire will utilize the same methodology to calculate the anti-imiglucerase ECL cut point.

Final Report Submission: May 31, 2010

- 1600-02 Shire commits to revise the cut point for the confirmatory anti-velaglucerase and anti-imiglucerase screening assays to a level that is less than or equal to the cut point of the screening assay.

Final Report Submission: May 31, 2010

- 1600-03 Shire commits to re-assess the IgE cut point for the current ECL methodology using a chemically synthesized hybrid control. Shire commits to support assay validation using patient baseline values.

Final Report Submission: May 31, 2010

- 1600-04 Shire commits to develop an assay to measure the ability of patient antibodies to block the uptake of velaglucerase and imiglucerase into target cells.

Final Report Submission: November 30, 2010

- 1600-05 Shire commits to re-analyze all archived pharmacokinetic (PK) samples for Study TKT032 (using adequate in-process quality controls and standard curves) and recalculate velaglucerase alfa PK parameters.

Study Completion Date: May 31, 2010
Final Report Submission: June 30, 2010

- 1600-06 Shire commits to conduct a prospective PK study in patients with Type 1 Gaucher disease in the case that Shire fails to adequately characterize velaglucerase alfa PK using the archived PK samples (discussed under PMC #1600-05 above).

Final Protocol Submission: December 31, 2010
Study Completion Date: March 31, 2013
Final Report Submission: September 30, 2013

POSTMARKETING COMMITMENTS NOT SUBJECT TO THE REPORTING REQUIREMENTS UNDER SECTION 506B

We remind you of your postmarketing commitments in your submission dated February 25, 2010. These commitments are listed below.

- 1600-07 Shire commits to develop and implement a kinetic assay with a physiologically relevant substrate for drug substance and drug product release and stability testing. Results and specifications will be included in the final report.

Final Report Submission: December 31, 2011

- 1600-08 Shire commits to develop and implement a quantitative method that measures total carbohydrate content. Results and specifications will be included in the final report.

Final Report Submission: February 28, 2011

- 1600-09 Shire commits to replace the non-quantitative SDS-PAGE Silver stain method with a quantitative SDS-PAGE Coomassie test. Results and specifications will be included in the final report.

Final Report Submission: February 28, 2011

- 1600-10 Shire commits to demonstrate that Long R3 IGF1 is well controlled to ensure no impact on product quality. The results will be included in the final report.

Final Report Submission: February 28, 2011

- 1600-11 Shire commits to demonstrate the clearance capability of the process to remove hydrocortisone through hydrocortisone spike studies. The results will be included in the final report.

Final Report Submission: November 30, 2010

- 1600-12 Shire commits to re-evaluate drug substance and drug product release and stability specifications. Shire will submit the revised specifications and supporting data in the final report.

Final Report Submission: December 31, 2011

- 1600-13 Shire commits to update the specifications for SEC, RP-HPLC, and the glycan map, and to include acceptance criteria for the leading shoulder in SEC-HPLC, for peaks A and B in RP-HPLC, and for peak group 2 in the glycan map.

Final Report Submission: July 1, 2010

- 1600-14 Shire commits to update the peptide map specification using new acceptance criteria to reflect control of impurities. Shire commits to add the peptide map as a drug substance and drug product release and stability test with the new acceptance criteria.

Final Report Submission: July 1, 2010

- 1600-15 Shire commits to include the cellular uptake bioassay for drug product release testing.

Final Report Submission: April 1, 2010

- 1600-16 Shire commits to provide a report containing the sub-visible particulates (2 – 10 μ m) analyses, risk assessment and risk mitigation strategies.

Final Report Submission: September 30, 2010

- 1600-17 Shire commits to include drug substance and drug product stress conditions in the annual stability program. The revised stability protocols will be included.

Final Protocol Submission: April 1, 2010

- 1600-18 Shire commits to evaluate the impact of pH on the in-use stability of the drug product and to provide assurance that procedures are in place to control this risk to product quality.

Final Protocol Submission: December 31, 2010

Submit clinical protocols to your IND 061220 for this product. Submit nonclinical and chemistry, manufacturing, and controls protocols and all study final reports to this NDA. In addition, under 21 CFR 314.81(b)(2)(vii) and 314.81(b)(2)(viii) you should include a status summary of each commitment in your annual report to this NDA. The status summary should include expected summary completion and final report submission dates, any changes in plans since the last annual report, and, for clinical studies/trials, number of patients entered into each study/trial. All submissions, including supplements, relating to these postmarketing commitments should be prominently labeled "Postmarketing Commitment Protocol," "Postmarketing Commitment Final Report," or "Postmarketing Commitment Correspondence."

PROMOTIONAL MATERIALS

You may request advisory comments on proposed introductory advertising and promotional labeling. To do so, submit, in triplicate, a cover letter requesting advisory comments, the proposed materials in draft or mock-up form with annotated references, and the package insert to:

Food and Drug Administration
Center for Drug Evaluation and Research
Division of Drug Marketing, Advertising, and Communications
5901-B Ammendale Road
Beltsville, MD 20705-1266

As required under 21 CFR 314.81(b)(3)(i), you must submit final promotional materials, and the package insert, at the time of initial dissemination or publication, accompanied by a Form FDA 2253. For instruction on completing the Form FDA 2253, see page 2 of the Form. For more information about submission of promotional materials to the Division of Drug Marketing, Advertising, and Communications (DDMAC), see <http://www.fda.gov/AboutFDA/CentersOffices/CDER/ucm090142.htm>.

Please submit one market package of the drug product when it is available.

LETTERS TO HEALTH CARE PROFESSIONALS

If you issue a letter communicating important safety-related information about this drug product (i.e., a "Dear Health Care Professional" letter), we request that you submit an electronic copy of the letter to both this NDA and to the following address:

MedWatch
Food and Drug Administration
Suite 12B-05
5600 Fishers Lane
Rockville, MD 20857

REPORTING REQUIREMENTS

We remind you that you must comply with reporting requirements for an approved NDA (21 CFR 314.80 and 314.81).

MEDWATCH-TO-MANUFACTURER PROGRAM

The MedWatch-to-Manufacturer Program provides manufacturers with copies of serious adverse event reports that are received directly by the FDA. New molecular entities and important new biologics qualify for inclusion for three years after approval. Your firm is eligible to receive copies of reports for this product. To participate in the program, please see the enrollment instructions and program description details at

<http://www.fda.gov/Safety/MedWatch/HowToReport/ucm166910.htm>.

If you have any questions, call Wes Ishihara, Regulatory Project Manager, at (301) 796-0069.

Sincerely,

{See appended electronic signature page}

Julie Beitz, M.D.

Director

Office of Drug Evaluation III

Center for Drug Evaluation and Research

Enclosure: Package Insert

HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use VPRIV safely and effectively. See full prescribing information for VPRIV.

VPRIV™ (velaglu­cerase alfa for injection)
Initial U.S. Approval: 2010

INDICATIONS AND USAGE

VPRIV (velaglu­cerase alfa for injection) is a hydrolytic lysosomal glucocerebrosi­de-specific enzyme indicated for long-term enzyme replacement therapy (ERT) for pediatric and adult patients with type 1 Gaucher disease (1).

DOSAGE AND ADMINISTRATION

- 60 Units/kg administered every other week as a 60-minute intravenous infusion (2).
- Patients currently being treated with imigluc­erase for Gaucher disease can be switched to VPRIV. Patients previously treated on a stable dose of imigluc­erase are recommended to begin treatment with VPRIV at that same dose when they switch from imigluc­erase to VPRIV (2).
- Physicians can make dosage adjustments based on achievement and maintenance of each patient's therapeutic goals. Clinical trials have evaluated doses ranging from 15 Units/kg to 60 Units/kg every other week (2).

DOSAGE FORMS AND STRENGTHS

- Lyophilized powder to be reconstituted and diluted for infusion (3).
- Available in 200 Units and 400 Units single-use vials (3).

CONTRAINDICATIONS

- None (4).

WARNINGS AND PRECAUTIONS

- Hypersensitivity reactions: Treatment with VPRIV should be carefully re-evaluated in the presence of significant evidence of hypersensitivity to the product (5.1).
- Infusion-related reactions (5.2).

ADVERSE REACTIONS

- The most common adverse reactions during clinical studies were infusion-related reactions (5.2, 6.1).
- Other commonly observed adverse reactions in ≥ 10% of patients were: headache, dizziness, abdominal pain, nausea, back pain, joint pain, upper respiratory tract infection, activated PTT prolonged, fatigue/asthenia, and pyrexia (6.1).

To report SUSPECTED ADVERSE REACTIONS, contact Shire Human Genetic Therapies, Inc. at the OnePathSM phone # 1-866-888-0660 or MedInfoGlobal@Shire.com, or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch

See Section 17 for PATIENT COUNSELING INFORMATION.

Revised: 02/2010

FULL PRESCRIBING INFORMATION: CONTENTS*

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*Sections or subsections omitted from the full prescribing information are not listed.

VPRIV™ (velaglucerase alfa for injection)

FULL PRESCRIBING INFORMATION

1 INDICATIONS AND USAGE

VPRIV (velaglucerase alfa for injection) is a hydrolytic lysosomal glucocerebroside-specific enzyme indicated for long-term enzyme replacement therapy (ERT) for pediatric and adult patients with type 1 Gaucher disease.

2 DOSAGE AND ADMINISTRATION

2.1 Recommended Dose

The recommended dose is 60 Units/kg administered every other week as a 60-minute intravenous infusion.

Patients currently being treated with imiglucerase for type 1 Gaucher disease may be switched to VPRIV. Patients previously treated on a stable dose of imiglucerase are recommended to begin treatment with VPRIV at that same dose when they switch from imiglucerase to VPRIV.

Dosage adjustments can be made based on achievement and maintenance of each patient's therapeutic goals. Clinical studies have evaluated doses ranging from 15 Units/kg to 60 Units/kg every other week.

VPRIV should be administered under the supervision of a healthcare professional.

2.2 Preparation and Administration Instructions

Use aseptic technique

VPRIV is a lyophilized powder, which requires reconstitution and dilution, and is intended for intravenous infusion only. VPRIV contains no preservatives and vials are single-use only. Discard any unused solution. VPRIV should be prepared as follows:

Determine the number of vials to be reconstituted based on the individual patient's weight and the prescribed dose. Follow the instructions in Table 1 for reconstitution.

Table 1: Reconstitution Instructions

	200 Units/vial	400 Units/vial
Volume of Sterile Water for Injection, USP, for reconstitution	2.2 mL	4.3 mL
Concentration after reconstitution	100 Units/mL	100 Units/mL
Withdrawal volume	2 mL	4 mL

Upon reconstitution, mix vials gently. **DO NOT SHAKE.** Prior to further dilution, visually inspect the solution in the vials; the solution should be clear to slightly opalescent and colorless; do not use if the solution is discolored or if foreign particulate matter is present. Withdraw the calculated volume of drug from the appropriate number of vials and dilute the total volume required in 100 mL of 0.9% sodium chloride solution suitable for IV administration. Mix gently. **DO NOT SHAKE.**

VPRIV should be administered over 60 minutes. VPRIV should not be infused with other products in the same infusion tubing as the compatibility in solution with other products has not been evaluated. The diluted solution should be filtered through an in-line low protein-binding 0.2µm filter during administration.

As VPRIV contains no preservatives, once reconstituted the product should be used immediately. If immediate use is not possible, the reconstituted or diluted product may be stored for up to 24 hours at 2 to 8°C (36 to 46°F). Do not freeze. Protect from light. The infusion should be completed within 24 hours of reconstitution of vials.

3 DOSAGE FORMS AND STRENGTHS

VPRIV is a sterile, white to off-white, lyophilized powder for reconstitution with Sterile Water for Injection, USP, to yield a final concentration of 100 Units/mL.

VPRIV is available as 200 Units and 400 Units single-use vials.

4 CONTRAINDICATIONS

None.

5 WARNINGS AND PRECAUTIONS

5.1 Hypersensitivity Reactions

Hypersensitivity reactions have been reported in patients in clinical studies with VPRIV [see *Adverse Reactions (6.1)*]. As with any intravenous protein product, hypersensitivity reactions are possible, therefore appropriate medical support should be readily available when VPRIV is administered. If a severe reaction occurs, current medical standards for emergency treatment are to be followed.

Treatment with VPRIV should be approached with caution in patients who have exhibited symptoms of hypersensitivity to the active ingredient or excipients in the drug product or to other enzyme replacement therapy.

5.2 Infusion-related Reactions

Infusion-related reactions were the most commonly observed adverse reactions in patients treated with VPRIV in clinical studies. The most commonly observed symptoms of infusion-related reactions were: headache, dizziness, hypotension, hypertension, nausea, fatigue/asthenia, and pyrexia. Generally the infusion-related reactions were mild and, in treatment-naïve patients, onset occurred mostly during the first 6 months of treatment and tended to occur less frequently

with time.

The management of infusion-related reactions should be based on the severity of the reaction, e.g. slowing the infusion rate, treatment with medications such as antihistamines, antipyretics and/or corticosteroids, and/or stopping and resuming treatment with increased infusion time.

Pre-treatment with antihistamines and/or corticosteroids may prevent subsequent reactions in those cases where symptomatic treatment was required. Patients were not routinely pre-medicated prior to infusion of VPRIV during clinical studies.

6 ADVERSE REACTIONS

6.1 Clinical Studies Experience

The data described below reflect exposure of 94 patients with type 1 Gaucher disease who received VPRIV at doses ranging from 15 Units/kg to 60 Units/kg every other week in 5 clinical studies. Fifty-four (54) patients were naïve to ERT and received VPRIV for 9 months and 40 patients switched from imiglucerase to VPRIV treatment and received VPRIV for 12 months [see *Clinical Studies (14)*]. Patients were between 4 and 71 years old at time of first treatment with VPRIV, and included 46 male and 48 female patients.

The most serious adverse reactions in patients treated with VPRIV were hypersensitivity reactions [see *Warnings and Precautions (5.1)*].

The most commonly reported adverse reactions (occurring in $\geq 10\%$ of patients) that were considered related to VPRIV are shown in Table 2. The most common adverse reactions were infusion-related reactions.

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice.

Table 2: Adverse Reactions Observed in $\geq 10\%$ of Patients with Type 1 Gaucher Disease Treated with VPRIV

System Organ Class Preferred Term	Naïve to ERT N = 54	Switched from imiglucerase to VPRIV N = 40
	Number of Patients (%)	
Nervous system disorders		
Headache	19 (35.2)	12 (30)
Dizziness	12 (22.2)	3 (7.5)
Gastrointestinal disorders		
Abdominal pain	10 (18.5)	6 (15)
Nausea	3 (5.6)	4 (10)
Musculoskeletal and connective tissue disorders		
Back pain	9 (16.7)	7 (17.5)
Joint pain (knee)	8 (14.8)	3 (7.5)
Infections and infestations		
Upper respiratory tract infection	17 (31.5)	12 (30)
Investigations		
Activated partial thromboplastin time prolonged	6 (11.1)	2 (5)
General disorders and administration site conditions		
Infusion-related reaction*	28 (51.9)	9 (22.5)
Pyrexia	12 (22.2)	5 (12.5)
Asthenia/Fatigue	7 (13)	5 (12.5)

*Denotes any event considered related to and occurring within up to 24 hours of VPRIV infusion

Less common adverse reactions affecting more than one patient ($>3\%$ in the treatment-naïve group and $>2\%$ in patients switched from imiglucerase to VPRIV treatment) were bone pain, tachycardia, rash, urticaria, flushing, hypertension, and hypotension.

Pediatric Patients

All adult adverse reactions to VPRIV are considered relevant to pediatric patients (ages 4 to 17 years). Adverse reactions more commonly seen in pediatric patients compared to adult patients include ($>10\%$ difference): upper respiratory tract infection, rash, aPTT prolonged; and pyrexia.

Immunogenicity

As with all therapeutic proteins, there is a potential for immunogenicity. In clinical studies, 1 of 54 treatment-naïve patients treated with VPRIV developed IgG class antibodies to VPRIV.

In this patient, the antibodies were determined to be neutralizing in an in vitro assay. No infusion-related reactions were reported for this patient. It is unknown if the presence of IgG antibodies to VPRIV is associated with a higher risk of infusion reactions. Patients with an immune response to other enzyme replacement therapies who are switching to VPRIV should continue to be monitored for antibodies.

Immunogenicity assay results are highly dependent on the sensitivity and specificity of the assay. Additionally, the observed incidence of antibody positivity in an assay may be influenced by several factors, including assay methodology, sample handling, timing of sample collection, concomitant medications, and underlying disease. For these reasons, comparison of the incidence of antibodies to VPRIV with the incidence of antibodies to other products may be misleading.

7 DRUG INTERACTIONS

No drug-drug interaction studies have been conducted.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy – Category B

Reproduction studies with velaglucerase alfa have been performed in pregnant rats at intravenous doses up to 17 mg/kg/day (102 mg/m²/day, about 1.8 times the recommended human dose of 60 Units/kg/day or 1.5 mg/kg/day or 55.5 mg/m²/day based on the body surface area). Reproduction studies have been performed in pregnant rabbits at intravenous doses up to 20 mg/kg/day (240 mg/m²/day, about 4.3 times the recommended human dose of 60 Units/kg/day based on the body surface area). These studies did not reveal any evidence of impaired fertility or harm to the fetus due to velaglucerase alfa.

A pre- and postnatal development study in rats showed no evidence of any adverse effect on pre- and postnatal development at doses up to 17 mg/kg (102 mg/m²/day, about 1.8 times the recommended human dose of 60 Units/kg/day based on the body surface area). There are, however, no adequate and well-controlled studies in pregnant women. Because animal reproduction studies are not always predictive of human response, VPRIV should be used during pregnancy only if clearly needed.

8.3 Nursing Mothers

There are no data from studies in lactating women. It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when VPRIV is administered to a nursing woman.

8.4 Pediatric Use

The safety and effectiveness of VPRIV have been established in patients between 4 and 17 years of age. Use of VPRIV in this age group is supported by evidence from adequate and well-controlled studies of VPRIV in adults and pediatric [20 of 94 (21%)] patients. The safety and efficacy profiles were similar between pediatric and adult patients [see *Adverse Reactions (6.1) and Clinical Studies (14)*]. The safety of VPRIV has not been established in pediatric patients.

younger than 4 years of age.

8.5 Geriatric Use

During clinical studies 4 patients aged 65 or older were treated with VPRIV. Clinical studies of VPRIV did not include sufficient numbers of subjects aged 65 and over to determine whether they respond differently from younger subjects. Other reported clinical experience has not identified differences in responses between the elderly and younger patients. In general, dose selection for an elderly patient should be approached cautiously, considering potential comorbid conditions.

10 OVERDOSAGE

There is no experience with overdose of VPRIV.

11 DESCRIPTION

The active ingredient of VPRIV is velaglucerase alfa, which is produced by gene activation technology in a human fibroblast cell line. Velaglucerase alfa is a glycoprotein of 497 amino acids; with a molecular weight of approximately 63 kDa. Velaglucerase alfa has the same amino acid sequence as the naturally occurring human enzyme, glucocerebrosidase. Velaglucerase alfa contains 5 potential N-linked glycosylation sites; four of these sites are occupied by glycan chains. Velaglucerase alfa is manufactured to contain predominantly high mannose-type N-linked glycan chains. The high mannose type N-linked glycan chains are specifically recognized and internalized via the mannose receptor present on the surface on macrophages, the cells that accumulate glucocerebroside in Gaucher disease. Velaglucerase alfa catalyzes the hydrolysis of the glycolipid glucocerebroside to glucose and ceramide in the lysosome.

VPRIV is dosed by Units/kg, where one Unit of enzyme activity is defined as the quantity of enzyme required to convert one micromole of p-nitrophenyl β -D-glucopyranoside to p-nitrophenol per minute at 37°C.

VPRIV is supplied as a sterile, preservative free, lyophilized powder in single-use vials. Following reconstitution with Sterile Water for Injection, USP, the solution contains the components listed in Table 3.

Table 3: VPRIV Composition Following Reconstitution

	Extractable 200 Units/vial	Extractable 400 Units/vial
Active Ingredient		
velaglucerase alfa	200 Units	400 Units
Inactive Ingredients		
citric acid, monohydrate	2.52 mg	5.04 mg
polysorbate 20	0.22 mg	0.44 mg
sodium citrate, dihydrate	25.88 mg	51.76 mg
sucrose	100 mg	200 mg

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

Gaucher disease is an autosomal recessive disorder caused by mutations in the GBA gene, which results in a deficiency of the lysosomal enzyme beta-glucocerebrosidase. Glucocerebrosidase catalyzes the conversion of the sphingolipid glucocerebroside into glucose and ceramide. The enzymatic deficiency causes an accumulation of glucocerebroside primarily in the lysosomal compartment of macrophages, giving rise to foam cells or "Gaucher cells". In this lysosomal storage disorder (LSD), clinical features are reflective of the accumulation of Gaucher cells in the liver, spleen, bone marrow, and other organs. The accumulation of Gaucher cells in the liver and spleen leads to organomegaly. Presence of Gaucher cells in the bone marrow and spleen lead to clinically significant anemia and thrombocytopenia.

Velaglucerase alfa catalyzes the hydrolysis of glucocerebroside, reducing the amount of accumulated glucocerebroside.

12.3 Pharmacokinetics

In a multicenter study conducted in pediatric (N=7, 4 to 17 years old) and adult (N=15, 19 to 62 years old) patients with type 1 Gaucher disease, pharmacokinetic evaluations were performed at Weeks 1 and 37 following 60-minute intravenous infusions of VPRIV 60 Units/kg every other week. Serum velaglucerase alfa concentrations declined rapidly with a mean half life of 11 to 12 minutes. The mean velaglucerase alfa clearance ranged from 6.72 to 7.56 mL/min/kg. The mean volume of distribution at steady state ranged from 82 to 108 mL/kg (8.2% to 10.8% of body weight). However, because an inadequately validated analytical assay method was used in the evaluations, the accurate and definitive pharmacokinetic parameter values are not currently available.

No accumulation or change in velaglucerase alfa pharmacokinetics over time from Weeks 1 to 37 was observed upon multiple-dosing 60 Units/kg every other week.

Based on the limited data, there were no notable pharmacokinetic differences between male and female patients in this study. The effect of age on pharmacokinetics of velaglucerase alfa was inconclusive.

The effect of anti-drug antibody formation on the pharmacokinetic parameters of velaglucerase alfa is unknown.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

Long-term studies in animals to evaluate carcinogenic potential or studies to evaluate mutagenic potential have not been performed with velaglucerase alfa.

In a male and female fertility study in rats, velaglucerase alfa did not cause any significant adverse effect on male or female fertility parameters up to a maximum dose of 17 mg/kg/day (102 mg/m²/day, about 1.8 times the recommended human dose of 60 Units/kg/day based on the

body surface area).

14 CLINICAL STUDIES

The efficacy of VPRIV was assessed in three clinical studies in a total of 99 patients with type I Gaucher disease: 82 patients age 4 years and older received VPRIV and 17 patients age 3 years and older received imiglucerase. Studies I and II were conducted in patients who were not currently receiving Gaucher disease-specific therapy. Study III was conducted in patients who were receiving imiglucerase treatment immediately before starting VPRIV. In these studies, VPRIV was administered intravenously over 60 minutes at doses ranging from 15 Units/kg to 60 Units/kg every other week.

14.1 Studies of VPRIV as Initial Therapy

Study I was a 12-month, randomized, double-blind, parallel-dose-group, multinational study in 25 patients age 4 years and older with Gaucher disease-related anemia and either thrombocytopenia or organomegaly. Patients were not allowed to have had disease-specific therapy for at least the previous 30 months; all but one had no prior therapy. The mean age was 26 years and 60% were male. Patients were randomized to receive VPRIV at a dose of either 45 Units/kg (N=13) or 60 Units/kg (N=12) every other week.

At baseline, mean hemoglobin concentration was 10.6 g/dL, mean platelet count was $97 \times 10^9/L$, mean liver volume was 3.6 % of body weight (% BW), and mean spleen volume was 2.9 % BW. For all studies, liver and spleen volumes were measured by MRI. The changes in clinical parameters after 12 months of treatment are shown in Table 4. The observed change from baseline in the primary endpoint, hemoglobin concentration, was considered to be clinically meaningful in light of the natural history of untreated Gaucher disease.

Table 4: Mean Change from Baseline to Month 12 for Clinical Parameters in Patients with Type 1 Gaucher Disease Initiating Therapy with VPRIV in Study I

Clinical Parameter	Mean Changes from Baseline \pm Std. Err. of the Mean	
	VPRIV Dose (given every other week)	
	45 Units/kg N = 13	60 Units/kg N = 12
Hemoglobin concentration change (g/dL)	$2.4 \pm 0.4^*$	$2.4 \pm 0.3^{**}$
Platelet count change ($\times 10^9/L$)	$41 \pm 14^*$	$51 \pm 12^*$
Liver volume change (% BW)	-0.30 ± 0.29	-0.84 ± 0.33
Spleen volume change (% BW)	$-1.9 \pm 0.6^*$	$-1.9 \pm 0.5^*$

** Primary study endpoint was hemoglobin concentration change in the 60 Unit/kg group, $p < 0.001$

* Statistically significant changes from baseline after adjusting for performing multiple tests

Study II was a 9-month, randomized, double-blind, active-controlled (imiglucerase), parallel-group, multinational study in 34 patients age 3 years and older. Patients were required to have Gaucher disease-related anemia and either thrombocytopenia or organomegaly. Patients were

not allowed to have had disease-specific therapy for at least the previous 12 months. The mean age was 30 years and 53% were female; the youngest patient who received VPRIV was age 4 years. Patients were randomized to receive either 60 Units/kg of VPRIV (N=17) or 60 Units/kg of imiglucerase (N=17) every other week.

At baseline, the mean hemoglobin concentration was 11.0 g/dL, mean platelet count was $171 \times 10^9/L$, and mean liver volume was 4.3 % BW. For the patients who had not had splenectomy (7 in each group) the mean spleen volume was 3.4 % BW. After 9 months of treatment, the mean absolute increase from baseline in hemoglobin concentration was $1.6 \text{ g/dL} \pm 0.2 \text{ (SE)}$ for patients treated with VPRIV. The mean treatment difference in change from baseline to 9 months [VPRIV – imiglucerase] was $0.1 \text{ g/dL} \pm 0.4 \text{ (SE)}$.

In Studies I and II, examination of age and gender subgroups did not identify differences in response to VPRIV among these subgroups. The number of non-Caucasian patients in these studies was too small to adequately assess any difference in effects by race.

14.2 Study in Patients Switching from Imiglucerase Treatment to VPRIV

Study III was a 12-month, open-label, single-arm, multinational study in 40 patients age 9 years and older who had been receiving treatment with imiglucerase at doses ranging between 15 Units/kg to 60 Units/kg for a minimum of 30 consecutive months. Patients also were required to have a stable biweekly dose of imiglucerase for at least 6 months prior to enrollment. The mean age was 36 years and 55% were female. Imiglucerase therapy was stopped, and treatment with VPRIV was administered every other week at the same number of units as the patient's previous imiglucerase dose. Adjustment of dosage was allowed by study criteria if needed in order to maintain clinical parameters.

Hemoglobin concentrations and platelet counts remained stable on average through 12 months of VPRIV treatment. After 12 months of treatment with VPRIV the median hemoglobin concentration was 13.5 g/dL (range: 10.8, 16.1) vs. the baseline value of 13.8 g/dL (range: 10.4, 16.5), and the median platelet count after 12 months was $174 \times 10^9/L$ (range: 24, 408) vs. the baseline value of $162 \times 10^9/L$ (range: 29, 399). No patient required dosage adjustment during the 12-month treatment period.

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16 HOW SUPPLIED/STORAGE AND HANDLING

VPRIV is a sterile, preservative free, lyophilized powder requiring reconstitution and further dilution prior to use. It is supplied in individually packaged glass vials, which are closed with a butyl rubber stopper with a fluoro-resin coating and are sealed with an aluminum overseal with a flip-off plastic cap. The vials are intended for single use only. VPRIV is available as: 200 Units/vial NDC 54092-701-02 and 400 Units/vial NDC 54092-701-04.

16.1 Storage

VPRIV should be stored in a refrigerator at 2 to 8°C (36 to 46°F). Do not use VPRIV after the expiration date on the vial. Do not freeze.

Protect vial from light.

17 PATIENT COUNSELING INFORMATION

- VPRIV should be administered under the supervision of a healthcare professional. VPRIV is a treatment that is given intravenously (by IV) every other week. The infusion typically takes up to 60 minutes.
- Patients should be advised that VPRIV may cause hypersensitivity reactions or infusion-related reactions. Infusion-related reactions can usually be managed by slowing the infusion rate, treatment with medications such as antihistamines, antipyretics and/or corticosteroids, and/or stopping and resuming treatment with increased infusion time. Pre-treatment with antihistamines and/or corticosteroids may prevent subsequent reactions. Treatment with VPRIV should be carefully re-evaluated in the presence of significant evidence of hypersensitivity to the product [*see Warnings and Precautions (5.1, 5.2)*].

Rx Only

VPRIV is manufactured by:

Shire Human Genetic Therapies, Inc.
700 Main Street
Cambridge, MA 02139

OnePath is a service mark and VPRIV is a trademark of Shire Human Genetic Therapies, Inc.

Application
Type/Number

Submission
Type/Number

Submitter Name

Product Name

NDA-22575

ORIG-1

SHIRE HUMAN
GENETIC
THERAPIES INC

VELAGLUCERASE ALFA

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/s/

JULIE G BEITZ
02/26/2010

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

**Assignee: Shire Human Genetic Therapies,
Inc.**

**Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS**

Attachment D

U.S. Patent No. 7,138,262 B1



US007138262B1

(12) **United States Patent**
Daniel(10) **Patent No.:** **US 7,138,262 B1**
(45) **Date of Patent:** **Nov. 21, 2006**(54) **HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH MANNOSE PROTEINS**(75) **Inventor:** Peter Francis Daniel, Natick, MA (US)(73) **Assignee:** Shire Human Genetic Therapies, Inc., Cambridge, MA (US)(*) **Notice:** Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.(21) **Appl. No.:** 09/641,471(22) **Filed:** Aug. 18, 2000(51) **Int. Cl.**

C12N 9/26 (2006.01)

C12P 19/28 (2006.01)

C12N 5/02 (2006.01)

C12N 5/06 (2006.01)

C12N 15/67 (2006.01)

C07H 21/04 (2006.01)

(52) **U.S. Cl.** 435/201; 435/85; 435/325; 435/358; 435/463; 536/23.2(58) **Field of Classification Search** 435/201, 435/325, 68.1, 69.1, 72, 85, 358, 365, 463; 536/23.2

See application file for complete search history.

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(74) **Attorney, Agent, or Firm**—Fish & Richardson P.C.(57) **ABSTRACT**

The invention features a method of producing a high mannose glucocerebrosidase (hmGCB) which includes: providing a cell which is capable of expressing glucocerebrosidase (GCB), and allowing production of GCB having a precursor oligosaccharide under conditions which prevent the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB, to thereby produce an hmGCB preparation. Preferably, the condition which prevents the removal of at least one mannose residue distal to the pentasaccharide core is inhibition of a class 1 processing mannosidase and/or a class 2 processing mannosidase. The invention also features an hmGCB preparation and methods of using an hmGCB preparation.

63 Claims, 1 Drawing Sheet

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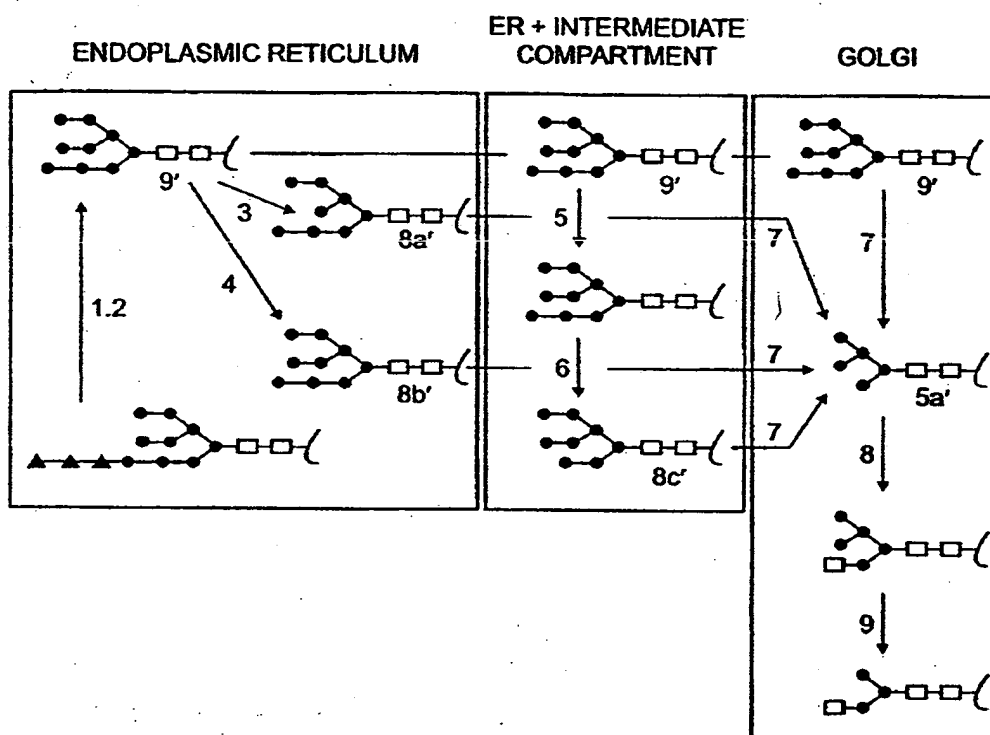


FIG. 1

1

HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH MANNOSE PROTEINS

BACKGROUND OF THE INVENTION

Gaucher disease is an autosomal recessive lysosomal storage disorder characterized by a deficiency in the lysosomal enzyme, glucocerebrosidase (GCB). GCB hydrolyzes the glycolipid glucocerebroside that is formed after degradation of glycosphingolipids in the membranes of white blood cells and red blood cells. The deficiency in this enzyme causes glucocerebroside to accumulate in large quantities in the lysosomes of phagocytic cells located in the liver, spleen and bone marrow of Gaucher patients. Accumulation of these molecules causes a range of clinical manifestations including splenomegaly, hepatomegaly, skeletal disorder, thrombocytopenia and anemia. (Beutler et al. Gaucher disease. In: The Metabolic and Molecular Bases of Inherited Disease (McGraw-Hill, Inc. New York, 1995) pp. 2625-2639)

Treatments for patients suffering from this disease include administration of analgesics for relief of bone pain, blood and platelet transfusions and, in some cases, splenectomy. Joint replacement is sometimes necessary for patients who experience bone erosion.

Enzyme replacement therapy with GCB has been used as a treatment for Gaucher disease. Current treatment of patients with Gaucher disease includes administration of a carbohydrate remodeled GCB derived from human placenta or Chinese hamster ovary (CHO) cells transfected with a GCB expression construct and known as alglucerase or imiglucerase, respectively. The treatment is extremely expensive in part because of the cost of removing sugars from GCB to expose the trimannosyl core of complex glycans in order to target the enzyme to mannose receptors on cells of reticuloendothelial origin. The scarcity of the human placental tissue (in the case of alglucerase), complex purification protocols, and the relatively large amounts of the carbohydrate remodeled GCB required all contribute to the cost of the treatment.

SUMMARY OF THE INVENTION

The invention is based, in part, on the discovery that by preventing removal of one or more mannose residues distal from the pentasaccharide core of a precursor oligosaccharide chain of a protein, e.g., a lysosomal storage enzyme, a high mannose protein such as high mannose glucocerebrosidase (hmGCB) can be obtained. These high mannose proteins can be used to target the protein to cells which express mannose receptors. Such cells can include cells of reticuloendothelial origin including macrophages, Kupffer cells and histiocytes. Thus, these high mannose proteins can be used, for example, to target delivery by receptor mediated endocytosis to lysosomes to treat various lysosomal storage diseases.

In particular, hmGCB has been found to efficiently target mannose receptors. Mannose receptors are present on macrophages and other cells, e.g., dendritic cells, cardiomyocytes and glial cells, and are instrumental in receptor-mediated endocytosis. The absence of GCB in patients with Gaucher disease leads to accumulation of glucocerebroside, primarily in cells of reticuloendothelial origin including macrophages, Kupffer cells and histiocytes. Because these cells express mannose receptors on their surface, hmGCB can be used to effectively target delivery of a corrective enzyme to the lysosomes through receptor-mediated

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endocytosis, thereby treating Gaucher disease. Surprisingly, it was found that hmGCB uptake by macrophages was increased as compared to uptake of GCB secreted from cells.

Accordingly, in one aspect, the invention features a method of producing a preparation of high mannose glucocerebrosidase (hmGCB). The method includes:

providing a cell which is capable of expressing GCB; and allowing production of GCB having a precursor oligosaccharide under conditions which prevent the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB, to thereby produce an hmGCB preparation.

In a preferred embodiment, the GCB is human GCB. In a preferred embodiment, the cell is a human cell.

In a preferred embodiment, the removal of: one or more α 1.2 mannose residue(s) distal to the pentasaccharide core is prevented; an α 1.3 mannose residue distal to the pentasaccharide core is prevented; and/or an α 1.6 mannose residue distal to the pentasaccharide core is prevented. Preferably, the removal of one or more α 1.2 mannose residue(s) distal to the pentasaccharide core is prevented.

In a preferred embodiment, the method can include contacting the cell with a substance which prevents the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB, e.g., prevents removal of one or more α 1.2-mannose residue(s) distal to the pentasaccharide core, an α 1.3 mannose residue distal to the pentasaccharide core and/or an α 1.6 mannose residue distal to the pentasaccharide core. Preferably, the removal of one or more α 1.2 mannose(s) residue distal to the pentasaccharide core is prevented.

In a preferred embodiment, the method includes contacting the cell with a substance which prevents the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB, wherein the substance is a mannosidase inhibitor. The mannosidase inhibitor can be a class 1 processing mannosidase inhibitor, a class 2 processing mannosidase inhibitor or both. The class 1 processing mannosidase inhibitor can be one or more of: kifunensine, deoxymannojirimycin, or a similar inhibitor. Preferably, the class 1 processing mannosidase inhibitor is kifunensine. Useful class 2 processing mannosidase inhibitors can include one or more of: swainsonine, mannostatin, 6-deoxy-1,4-dideoxy-1,4-imino-D-mannitol (6-deoxy-DIM), and 6-deoxy-6-fluoro-1,4-dideoxy-1,4-imino-D-mannitol (6-deoxy-6-fluoro-DIM). Preferably, the class 2 processing mannosidase inhibitor is swainsonine.

In a preferred embodiment, a mannosidase inhibitor is present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml.

In a preferred embodiment, the method further includes contacting the cell with a class 1 processing mannosidase inhibitor and a class 2 processing mannosidase inhibitor. In a preferred embodiment, the class 1 processing mannosidase inhibitor is present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; the class 2 processing mannosidase inhibitor is present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; each of the class 1 processing and class 2 processing mannosidase inhibitors are present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; the total concentration of the class 1 processing and class 2 processing mannosidase inhibitors

present is between about 0.025 to 40.0 µg/ml, 0.05 to 20 µg/ml, 0.05 to 10 µg/ml, preferably between about 0.1 to 4.0 µg/ml.

In a preferred embodiment, the cell carries a mutation for, e.g., a knockout for, at least one Golgi processing mannosidase. The mutation can be one which reduces the expression of the gene, reduces protein or activity levels, or alters the distribution or other post translational modifications of the mannosidase, e.g., the processing of the carbohydrate chains. The mutation can be one which reduces the level of the Golgi processing mannosidase activity, e.g., one which reduces gene expression, e.g., a null mutation, e.g., a deletion, a frameshift or an insertion. In a preferred embodiment the mutation is a knockout, e.g., in the mannosidase gene. The mutation can affect the structure (and activity of the protein), and can, e.g., be a temperature sensitive mutation or a truncation. In a preferred embodiment, the cell carries a mutation, e.g., a knockout, for: a class 1 processing mannosidase; a class 2 processing mannosidase; a class 1 processing mannosidase and a class 2 processing mannosidase. In a preferred embodiment, the class 1 processing mannosidase is: Golgi mannosidase IA; Golgi mannosidase IB; Golgi mannosidase IC; or combinations thereof. In a preferred embodiment, the class 2 processing mannosidase is: Golgi mannosidase II.

In a preferred embodiment, the cell includes a nucleic acid sequence, such as an antisense molecule or ribozyme, which can bind to or inactivate a cellular mannosidase nucleic acid sequence, e.g., mRNA, and inhibit expression of the protein. In a preferred embodiment, the nucleic acid sequence is: a class 1 processing mannosidase antisense molecule; a class 2 processing mannosidase antisense molecule; both a class 1 processing mannosidase antisense molecule and a class 2 processing mannosidase antisense molecule. In a preferred embodiment, the class 1 processing mannosidase is: Golgi mannosidase IA; Golgi mannosidase IB; Golgi mannosidase IC; and combinations thereof. In a preferred embodiment, the class 2 processing mannosidase is: Golgi mannosidase II.

In a preferred embodiment, the cell includes a molecule, e.g., an exogenously supplied molecule, which binds and inhibits a mannosidase. The molecule can be, e.g., a single chain antibody, an intracellular protein or a competitive or non-competitive inhibitor.

In a preferred embodiment, the hmGCB molecule includes a carbohydrate chain having at least four mannose residues. For example, the molecule has at least one carbohydrate chain having five mannose residues, the hmGCB molecule has at least one carbohydrate chain having six mannose residues, the hmGCB molecule has at least one carbohydrate chain having seven mannose residues, the hmGCB molecule has at least one carbohydrate chain having eight mannose residues, the hmGCB molecule has at least one carbohydrate chain having nine mannose residues. Preferably, the hmGCB molecule has at least one carbohydrate chain having five, eight or nine mannose residues.

In a preferred embodiment, the hmGCB produced (either one or more hmGCB molecules or the preparation as a whole) has a ratio of mannose residues to GlcNAc residues which is greater than 3 mannose residues to 2 GlcNAc residues, preferably the ratio of mannose to GlcNAc is 4:2, 5:2, 6:2, 7:2, 8:2, 9:2; more preferably the ratio of mannose to GlcNAc is 5:2, 8:2 or 9:2.

In a preferred embodiment, the removal of one or more mannose residues distal to the pentasaccharide core is prevented on one, two, three or four of the carbohydrate chains of an hmGCB molecule.

In a preferred embodiment, at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or all of the hmGCB molecules of the preparation have at least one, and preferably two, three or four carbohydrate chains in which the removal of one or more mannose residues distal to the pentasaccharide core has been prevented.

In a preferred embodiment, the hmGCB preparation is a relatively heterogeneous preparation. Preferably, less than 80%, 70%, 60%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5% or 1% of the carbohydrate chains in the hmGCB preparation have the same number of mannose residues in addition to the pentasaccharide core. For example, the ratio of carbohydrate chains having the same number of mannose residues in addition to the pentasaccharide core to carbohydrate chains having a different number of mannose residues can be about: 60%:40%; 50%:50%; 40%:60%; 30%:70%; 25%:75%; 20%:80%; 15%:85%; 10%:90%; 5% or less:95% or more.

In a preferred embodiment, activity of Golgi mannosidase IA and/or IB and/or IC is inhibited and at least about 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or 100% of the carbohydrate chains in the hmGCB preparation have five or more mannose residues, e.g., five, six, seven, eight and/or nine mannose residues. In a preferred embodiment, activity of Golgi mannosidase I is inhibited and the ratio of carbohydrate chains having five or more mannose residues to carbohydrate chains having four or less mannose residues is about 60%:40%; 70%:30%; 75%:25%; 80%:20%; 85%:15%; 90%:10%; 95%:5%; 99%:1%; or 100%:0%.

In a preferred embodiment, activity of Golgi mannosidase II is inhibited and at least about 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or 100% of the carbohydrate chains in the hmGCB preparation have five or more mannose residues, e.g., five, six, seven, eight and/or nine mannose residues. In a preferred embodiment, activity of Golgi mannosidase II is inhibited and the ratio of carbohydrate chains having five or more mannose residues to carbohydrate chains having four or less mannose residues is about 60%:40%; 70%:30%; 75%:25%; 80%:20%; 85%:15%; 90%:10%; 95%:5%; 99%:1%; or 100%:0%.

In a preferred embodiment, the cell includes an exogenous nucleic acid sequence which includes a GCB coding region. In a preferred embodiment, the cell further includes a regulatory sequence, an endogenous or exogenous regulatory sequence, which functions to regulate expression of the exogenous GCB coding region.

In a preferred embodiment, the cell includes an exogenous regulatory sequence which functions to regulate expression of an endogenous GCB coding sequence, e.g., the regulatory sequence is integrated into the genome of the cell such that it regulates expression of an endogenous GCB coding sequence.

In a preferred embodiment, the regulatory sequence includes one or more of: a promoter, an enhancer, an upstream activating sequence (UAS), a scaffold-attachment region or a transcription factor-binding site. In a preferred embodiment, the regulatory sequence includes: a regulatory sequence from a metallothionein-I gene, e.g., a mouse metallothionein-I gene, a regulatory sequence from an SV-40 gene, a regulatory sequence from a cytomegalovirus gene, a regulatory sequence from a collagen gene, a regulatory sequence from an actin gene, a regulatory sequence from an immunoglobulin gene, a regulatory sequence from the HMG-CoA reductase gene, or a regulatory sequence from the EF-1 α gene.

In a preferred embodiment, the cell is: a eukaryotic cell. In a preferred embodiment, the cell is of fungal, plant or

animal origin, e.g., vertebrate origin. In a preferred embodiment, the cell is: a mammalian cell, e.g., a primary or secondary mammalian cell, e.g., a fibroblast, a hematopoietic stem cell, a myoblast, a keratinocyte, an epithelial cell, an endothelial cell, a glial cell, a neural cell, a cell comprising a formed element of the blood, a muscle cell and precursors of these somatic cells; a transformed or immortalized cell line. Preferably, the cell is a human cell. Examples of immortalized human cell lines useful in the present method include, but are not limited to: a Bowes Melanoma cell (ATCC Accession No. CRL 9607), a Daudi cell (ATCC Accession No. CCL 213), a HeLa cell and a derivative of a HeLa cell (ATCC Accession Nos. CCL2 CCL2.1 and CCL 2.2), a HL-60 cell (ATCC Accession No. CCL 240), an HT-1080 cell (ATCC Accession No. CCL 121), a Jurkat cell (ATCC Accession No. TIB 152), a KB carcinoma cell (ATCC Accession No. CCL 17), a K-562 leukemia cell (ATCC Accession No. CCL 243), a MCF-7 breast cancer cell (ATCC Accession No. BTH 22), a MOLT-4 cell (ATCC Accession No. 1582), a Namalwa cell (ATCC Accession No. CRL 1432), a Raji cell (ATCC Accession No. CCL 86), a RPMI 8226 cell (ATCC Accession No. CCL 155), a U-937 cell (ATCC Accession No. 1593), WI-28VA13 sub line 2R4 cells (ATCC Accession No. CCL 155), a CCRF-CEM cell (ATCC Accession No. CCL 119) and a 2780AD ovarian carcinoma cell (Van Der Blick et al., Cancer Res. 48:5927-5932, 1988), as well as hetero-hybridoma cells produced by fusion of human cells and cells of another species. In another embodiment, the immortalized cell line can be cell line other than a human cell line, e.g., a CHO cell line, a COS cell line. In another embodiment, the cell can be a from a clonal cell strain or clonal cell line.

In a preferred embodiment, a population of cells which are capable of expressing hmGCB is provided, and at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or all of the cells produce hmGCB molecules with at least one carbohydrate chain, and preferably two, three, or four carbohydrate chains, having the specified number of mannose residues.

In a preferred embodiment, the cell is cultured in culture medium which includes at least one mannosidase inhibitor. In a preferred embodiment, the method further includes obtaining the hmGCB from the medium in which the cell is cultured.

In another aspect, the invention features a method of producing a preparation of hmGCB. The method includes: providing a cell which is capable of expressing GCB; and allowing production of GCB having a precursor oligosaccharide under conditions which inhibit class 1 processing mannosidase activity and class 2 processing mannosidase activity such that the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB is prevented, to thereby produce an hmGCB preparation.

In a preferred embodiment, the GCB is human GCB. In a preferred embodiment, the cell is a human cell.

In a preferred embodiment, the removal of one or more α 1,2 mannose residue(s) distal to the pentasaccharide core is prevented; an α 1,3 mannose residue distal to the pentasaccharide core is prevented; and/or an α 1,6 mannose residue distal to the pentasaccharide core is prevented. Preferably, the removal of one or more α 1,2 mannose residue(s) distal to the pentasaccharide core is prevented.

In a preferred embodiment, the method can include: contacting the cell with a substance which inhibits a class 1 processing mannosidase activity and a substance which inhibits a class 2 processing mannosidase activity thereby

preventing the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB. In a preferred embodiment, the substances prevent removal of one or more α 1,2 mannose residue distal to the pentasaccharide core.

In a preferred embodiment, the method includes contacting the cell with a substance which inhibits a class 1 processing mannosidase activity and a substance which inhibits a class 2 processing mannosidase activity, wherein the substances are a class 1 processing mannosidase inhibitor and a class 2 processing mannosidase inhibitor. In a preferred embodiment, the class 1 processing mannosidase inhibitor can be one or more of: kifunensine, deoxymannojirimycin, or a similar inhibitor. Preferably, the class 1 processing mannosidase inhibitor is kifunensine. In a preferred embodiment, the class 2 processing mannosidase inhibitor can be one or more of: swainsonine, mannosatin, 6-deoxy-DIM, and 6-deoxy-6-fluoro-DIM. Preferably, the class 2 processing mannosidase inhibitor is swainsonine.

In a preferred embodiment, a class 1 processing mannosidase inhibitor is present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; a class 2 processing mannosidase inhibitor is present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; each of the class 1 processing and class 2 processing mannosidase inhibitors are present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; the total concentration of the class 1 processing and class 2 processing mannosidase inhibitors present is between about 0.025 to 40.0 μ g/ml, 0.05 to 20 μ g/ml, 0.05 to 10 μ g/ml, preferably between about 0.1 to 4.0 μ g/ml.

In a preferred embodiment, the cell carries a mutation for, e.g., a knockout for, a class 1 mannosidase and a class 2 mannosidase. The mutation can be one which reduces the expression of the gene, reduces protein or activity levels, or alters the distribution or other post translational modifications of the mannosidase, e.g., the processing of the carbohydrate chains. The mutation can be one which reduces the level of a class 1 processing mannosidase and/or a class 2 processing mannosidase activity, e.g., one which reduces gene expression, e.g., a null mutation, e.g., a deletion, a frameshift, or an insertion. In a preferred embodiment, the mutation is a knockout in the mannosidase gene. The mutation can affect the structure (and activity of the protein), and can, e.g., be a temperature sensitive mutant. In a preferred embodiment, the class 1 processing mannosidase is: Golgi mannosidase IA; Golgi mannosidase IB; Golgi mannosidase IC; combinations thereof. In a preferred embodiment, the class 2 processing mannosidase is: Golgi mannosidase II.

In a preferred embodiment, the cell includes both a class 1 processing mannosidase antisense molecule and a class 2 processing mannosidase antisense molecule. In a preferred embodiment, the class 1 processing mannosidase is: Golgi mannosidase IA; Golgi mannosidase IB; Golgi mannosidase IC; combinations thereof. In a preferred embodiment, the class 2 processing mannosidase is: Golgi mannosidase II.

In a preferred embodiment, the cell includes a molecule, e.g., an exogenously supplied molecule, which binds and inhibits a mannosidase. The molecule can be, e.g., a single chain antibody, an intracellular protein or a competitive or non-competitive inhibitor.

In a preferred embodiment, the class 1 processing mannosidase activity and the class 2 mannosidase activity can be

inhibited by different mechanisms. For example, a class 1 processing mannosidase activity can be inhibited by contacting the cell with a substrate which inhibits a class 1 processing mannosidase, e.g., a class 1 mannosidase inhibitor, and the class 2 processing mannosidase can be inhibited by using a cell which is a knockout of a class 2 mannosidase and/or includes a class 2 mannosidase antisense molecule. In another preferred embodiment, a class 2 processing mannosidase activity can be inhibited by contacting the cell with a substrate which inhibits a class 2 processing mannosidase, e.g., a class 2 mannosidase inhibitor, and the class 1 processing mannosidase can be inhibited by using a cell which is a knockout of a class 1 mannosidase and/or includes a class 1 mannosidase antisense molecule.

In a preferred embodiment, the hmGCB molecule includes a carbohydrate chain having at least four mannose residues. For example, the hmGCB molecule has at least one carbohydrate chain having five mannose residues, the hmGCB molecule has at least one carbohydrate chain having six mannose residues, the hmGCB molecule has at least one carbohydrate chain having seven mannose residues, the hmGCB molecule has at least one carbohydrate chain having eight mannose residues, the hmGCB molecule has at least one carbohydrate chain having nine mannose residues. Preferably, the hmGCB molecule has at least one carbohydrate chain having five, eight or nine mannose residues.

In a preferred embodiment, the hmGCB produced (either one or more hmGCB molecules or the preparation as a whole) has a ratio of mannose residues to GlcNAc residues which is greater than 3 mannose residues to 2 GlcNAc residues, preferably the ratio of mannose to GlcNAc is 4:2, 5:2, 6:2, 7:2, 8:2, 9:2, more preferably the ratio of mannose to GlcNAc is 8:2 or 9:2.

In a preferred embodiment, the removal of one or more mannose residues distal to the pentasaccharide core is prevented on one, two, three or four of the carbohydrate chains of the hmGCB molecule.

In a preferred embodiment, at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or all of the hmGCB molecules of the preparation have at least one, and preferably two, three or four carbohydrate chains in which the removal of one or more mannose residues distal to the pentasaccharide core has been prevented.

In a preferred embodiment, the hmGCB preparation is a relatively heterogeneous preparation. Preferably, less than 80%, 70%, 60%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5% or 1% of the carbohydrate chains in the hmGCB preparation have the same number of mannose residues in addition to the pentasaccharide core. For example, the ratio of carbohydrate chains having the same number of mannose residues in addition to the pentasaccharide core to carbohydrate chains having a different number of mannose residues can be about: 60%:40%; 50%:50%; 40%:60%; 30%:70%; 25%:75%; 20%:80%; 15%:85%; 10%:90%; 5% or less:95% or more.

In a preferred embodiment, activity of a class 1 processing mannosidase, e.g., Golgi mannosidase IA and/or Golgi mannosidase IB and/or Golgi mannosidase IC, and activity of a class 2 processing mannosidase, e.g., Golgi mannosidase II, are inhibited and at least about 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or 100% of the carbohydrate chains in the hmGCB preparation have five or more mannose residues, e.g., five, six, seven, eight and/or nine mannose residues. In a preferred embodiment, activity of a class 1 processing mannosidase, e.g., Golgi mannosidase IA and/or Golgi mannosidase IB and/or Golgi mannosidase IC, and activity of a class 2 processing mannosidase, e.g., Golgi

mannosidase II, are inhibited and the ratio of carbohydrate chains having five or more mannose residues to carbohydrate chains having four or less mannose residues, respectively, is about 60%:40%; 70%:30%; 75%:25%; 80%:20%; 85%:15%; 90%:10%; 95%:5%; 99%:1%; or 100%:0%.

In a preferred embodiment, the cell includes an exogenous nucleic acid sequence which includes a GCB coding region. In a preferred embodiment, the cell further includes a regulatory sequence, an endogenous or exogenous regulatory sequence, which functions to regulate expression of the exogenous GCB coding region.

In a preferred embodiment, the cell includes an exogenous regulatory sequence which functions to regulate expression of an endogenous GCB coding sequence, e.g., the regulatory sequence is integrated into the genome of the cell such that it regulates expression of an endogenous GCB coding sequence.

In a preferred embodiment, the regulatory sequence includes one or more of: a promoter, an enhancer, an upstream activating sequence (UAS), a scaffold-attachment region or a transcription factor-binding site. In a preferred embodiment, the regulatory sequence includes: a regulatory sequence from a metallothionein-I gene, e.g., a mouse metallothionein-I gene, a regulatory sequence from an SV-40 gene, a regulatory sequence from a cytomegalovirus gene, a regulatory sequence from a collagen gene, a regulatory sequence from an actin gene, a regulatory sequence from an immunoglobulin gene, a regulatory sequence from the HMG-CoA reductase gene, or a regulatory sequence from the EF-1 α gene.

In a preferred embodiment, the cell is: a eukaryotic cell. In a preferred embodiment, the cell is of fungal, plant or animal origin, e.g., vertebrate origin. In a preferred embodiment, the cell is: a mammalian cell, e.g., a primary or secondary mammalian cell, e.g., a fibroblast, a hematopoietic stem cell, a myoblast, a keratinocyte, an epithelial cell, an endothelial cell, a glial cell, a neural cell, a cell comprising a formed element of the blood, a muscle cell and precursors of these somatic cells; a transformed or immortalized cell line. Preferably, the cell is a human cell. Examples of immortalized human cell lines useful in the present method include, but are not limited to: a Bowes Melanoma cell (ATCC Accession No. CRL 9607), a Daudi cell (ATCC Accession No. CCL 213), a HeLa cell and a derivative of a HeLa cell (ATCC Accession Nos. CCL2 CCL2.1 and CCL 2.2), a HI-60 cell (ATCC Accession No. CCL 240), an HT-1080 cell (ATCC Accession No. CCL 121), a Jurkat cell (ATCC Accession No. TIB 152), a KB carcinoma cell (ATCC Accession No. CCL 17), a K-562 leukemia cell (ATCC Accession No. CCL 243), a MCF-7 breast cancer cell (ATCC Accession No. BTH 22), a MOLT-4 cell (ATCC Accession No. 1582), a Namalwa cell (ATCC Accession No. CRL 1432), a Raji cell (ATCC Accession No. CCL 86), a RPMI 8226 cell (ATCC Accession No. CCL 155), a U-937 cell (ATCC Accession No. 1593), WI-28VA13 sub line 2R4 cells (ATCC Accession No. CCL 155), a CCRF-CEM cell (ATCC Accession No. CCL 119) and a 2780AD ovarian carcinoma cell (Van Der Blick et al., Cancer Res. 48:5927-5932, 1988), as well as heterohybridoma cells produced by fusion of human cells and cells of another species. In another embodiment, the immortalized cell line can be cell line other than a human cell line, e.g., a CHO cell line, a COS cell line. In another embodiment, the cell can be from a clonal cell strain or clonal cell line.

In a preferred embodiment, a population of cells which are capable of expressing hmGCB is provided, and at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%.

98%, 99% or all of the cells produce hmGCB with at least one carbohydrate chain, preferably two, three, or four carbohydrate chains, having the specified number of mannose residues.

In a preferred embodiment, the cell is cultured in a culture medium which includes at least one class 1 processing mannosidase inhibitor and at least one class 2 processing mannosidase inhibitor. In a preferred embodiment, the method further includes obtaining the hmGCB from the medium in which the cell is cultured.

In another aspect, the invention features a method of producing a preparation of hmGCB. The method includes: providing a cell into which a nucleic acid sequence comprising an exogenous regulatory sequence has been introduced such that the regulatory sequence regulates the expression of an endogenous GCB coding region; and

allowing production of GCB having a precursor oligosaccharide under conditions which prevent the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB, to thereby produce an hmGCB preparation.

In a preferred embodiment, the GCB is human GCB.

In a preferred embodiment, the removal of: one or more α 1,2 mannose residue(s) distal to the pentasaccharide core is prevented; an α 1,3 mannose residue distal to the pentasaccharide core is prevented; and/or an α 1,6 mannose residue distal to the pentasaccharide core is prevented. Preferably, the removal of one or more α 1,2 mannose residue(s) distal to the pentasaccharide core is prevented.

In a preferred embodiment, the method can include contacting the cell with a substance which prevents the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB, e.g., prevents removal of one or more α 1,2 mannose residue(s) distal to the pentasaccharide core, an α 1,3 mannose residue distal to the pentasaccharide core and/or an α 1,6 mannose residue distal to the pentasaccharide core. Preferably, the removal of one or more α 1,2 mannose(s) residue distal to the pentasaccharide core is prevented.

In a preferred embodiment, the method includes contacting the cell with a substance which prevents the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB, and the substance is a mannosidase inhibitor. The mannosidase inhibitor can be a class 1 processing mannosidase inhibitor, a class 2 processing mannosidase inhibitor or both. The class 1 processing mannosidase inhibitor can be one or more of: kifunensine, deoxymannojirimycin, or a similar inhibitor. Preferably, the class 1 processing mannosidase inhibitor is kifunensine. Useful class 2 processing mannosidase inhibitors can include one or more of: swainsonine, mannosidase, 6-deoxy-DIM, 6-deoxy-6-fluoro-DIM. Preferably, the class 2 processing mannosidase inhibitor is swainsonine.

In a preferred embodiment, a mannosidase inhibitor is present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml.

In a preferred embodiment, the method further includes contacting the cell with a class 1 processing mannosidase inhibitor and a class 2 processing mannosidase inhibitor. In a preferred embodiment, a class 1 processing mannosidase inhibitor is present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; a class 2 processing mannosidase inhibitor is present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; each of the class

1 processing and class 2 processing mannosidase inhibitors are present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; the total concentration of the class 1 processing and class 2 processing mannosidase inhibitors present is between about 0.025 to 40.0 μ g/ml, 0.05 to 20 μ g/ml, 0.05 to 10 μ g/ml, preferably between about 0.1 to 4.0 μ g/ml.

In a preferred embodiment, the cell carries a mutation for, e.g., a knockout for, at least one mannosidase. The mutation can be one which reduces the expression the gene, reduces protein or activity levels, or alters the distribution or other post translational modifications of the mannosidase, e.g., the processing of the carbohydrate chains. The mutation can be one which reduces the level of the Golgi processing mannosidase activity, e.g., one which reduces gene expression, e.g., a null mutation, e.g., a deletion, a frameshift or an insertion. In a preferred embodiment the mutation is a knockout, e.g., in the mannosidase gene. The mutation can affect the structure (and activity of the protein), and can, e.g., be a temperature sensitive mutation or a truncation. In a preferred embodiment, the cell carries a mutation, e.g., a knockout, for: a class 1 processing mannosidase; a class 2 processing mannosidase; a mutant, e.g., a knockout, for a class 1 processing mannosidase and a class 2 processing mannosidase. In a preferred embodiment, the class 1 processing mannosidase is: Golgi mannosidase IA; Golgi mannosidase IB; Golgi mannosidase IC; or combinations thereof. In a preferred embodiment, the class 2 processing mannosidase is: Golgi mannosidase II.

In a preferred embodiment, the cell includes a nucleic acid sequence, such as an antisense molecule or ribozyme, which can bind to or inactivate a cellular mannosidase nucleic acid sequence, e.g., mRNA, and inhibit expression of the protein.

In a preferred embodiment, the nucleic acid sequence is: a class 1 processing mannosidase antisense molecule; a class 2 processing mannosidase antisense molecule; both a class 1 processing mannosidase antisense molecule and a class 2 processing mannosidase antisense molecule. In a preferred embodiment, the class 1 processing mannosidase is: Golgi mannosidase IA; Golgi mannosidase IB; Golgi mannosidase IC; combinations thereof. In a preferred embodiment, the class 2 processing mannosidase is: Golgi mannosidase II.

In a preferred embodiment, the cell includes a molecule, e.g., an exogenously supplied molecule, which binds and inhibits a mannosidase. The molecule can be, e.g., a single chain antibody, an intracellular protein or a competitive or non-competitive inhibitor.

In a preferred embodiment, the hmGCB molecule includes a carbohydrate chain having at least four mannose residues. For example, the hmGCB molecule has at least one carbohydrate chain having five mannose residues, the hmGCB molecule has at least one carbohydrate chain having six mannose residues, the hmGCB molecule has at least one carbohydrate chain having seven mannose residues, the hmGCB molecule has at least one carbohydrate chain having eight mannose residues, the hmGCB molecule has at least one carbohydrate chain having nine mannose residues. Preferably, the hmGCB molecule has at least one carbohydrate chain having five, eight or nine mannose residues.

In a preferred embodiment, the hmGCB produced (either one or more hmGCB molecules or the preparation as a whole) has a ratio of mannose residues to GlcNAc residues which is greater than 3 mannose residues to 2 GlcNAc residues, preferably the ratio of mannose to GlcNAc is 4:2, 5:2, 6:2, 7:2, 8:2, 9:2, more preferably the ratio of mannose to GlcNAc is 5:2, 8:2 or 9:2.

In a preferred embodiment, the removal of one or more mannose residues distal to the pentasaccharide core is prevented on one, two, three or four of the carbohydrate chains of the hmGCB molecule.

In a preferred embodiment, at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or all of the hmGCB molecules of the preparation have at least one, and preferably two, three or four carbohydrate chains in which the removal of one or more mannose residues distal to the pentasaccharide core has been prevented.

In a preferred embodiment, the hmGCB preparation is a relatively heterogeneous preparation. Preferably, less than 80%, 70%, 60%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5% or 1% of the carbohydrate chains in the hmGCB preparation have the same number of mannose residues in addition to the pentasaccharide core. For example, the ratio of carbohydrate chains having the same number of mannose residues in addition to the pentasaccharide core to carbohydrate chains having a different number of mannose residues can be about: 60%:40%; 50%:50%; 40%:60%; 30%:70%; 25%:75%; 20%:80%; 15%:85%; 10%:90%; 5% or less:95% or more.

In a preferred embodiment, activity of Golgi mannosidase IA and/or IB and/or IC is inhibited and at least about 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or 100% of the carbohydrate chains in the hmGCB preparation have five or more mannose residues, e.g., five, six, seven, eight, and/or nine mannose residues. In a preferred embodiment, activity of Golgi mannosidase I is inhibited and the ratio of carbohydrate chains having five or more mannose residues to carbohydrate chains having four or less mannose residues is about 60%:40%; 70%:30%; 75%:25%; 80%:20%; 85%:15%; 90%:10%; 95%:5%; 99%:1%; or 100%:0%.

In a preferred embodiment, activity of Golgi mannosidase II is inhibited and at least about 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or 100% of the carbohydrate chains in the hmGCB preparation have five or more mannose residues. In a preferred embodiment, activity of Golgi mannosidase II is inhibited and the ratio of carbohydrate chains having five or more mannose residues to carbohydrate chains having four or less mannose residues is about 60%:40%; 70%:30%; 75%:25%; 80%:20%; 85%:15%; 90%:10%; 95%:5%; 99%:1%; or 100%:0%.

In a preferred embodiment, the regulatory sequence includes one or more of: a promoter, an enhancer, an upstream activating sequence (UAS), a scaffold-attachment region or a transcription factor-binding site. In a preferred embodiment, the regulatory sequence includes: a regulatory sequence from a metallothionein-I gene, e.g., a mouse metallothionein-I gene, a regulatory sequence from an SV-40 gene, a regulatory sequence from a cytomegalovirus gene, a regulatory sequence from a collagen gene, a regulatory sequence from an actin gene, a regulatory sequence from an immunoglobulin gene, a regulatory sequence from the HMG-CoA reductase gene, or a regulatory sequence from the EF-1 α gene.

In a preferred embodiment, the cell is: a eukaryotic cell. In a preferred embodiment, the cell is of fungal, plant or animal origin, e.g., vertebrate origin. In a preferred embodiment, the cell is: a mammalian cell, e.g., a primary or secondary mammalian cell, e.g., a fibroblast, a hematopoietic stem cell, a myoblast, a keratinocyte, an epithelial cell, an endothelial cell, a glial cell, a neural cell, a cell comprising a formed element of the blood, a muscle cell and precursors of these somatic cells; a transformed or immortalized cell line. Preferably, the cell is a human cell. Examples of immortalized human cell lines useful in the

present method include, but are not limited to: a Bowes Melanoma cell (ATCC Accession No. CRL 9607), a Daudi cell (ATCC Accession No. CCL 213), a HeLa cell and a derivative of a HeLa cell (ATCC Accession Nos. CCL2, CCL2.1 and CCL 2.2), a HL-60 cell (ATCC Accession No. CCL 240), an HT-1080 cell (ATCC Accession No. CCL 121), a Jurkat cell (ATCC Accession No. TIB 152), a KB carcinoma cell (ATCC Accession No. CCL 17), a K-562 leukemia cell (ATCC Accession No. CCL 243), a MCF-7 breast cancer cell (ATCC Accession No. BTH 22), a MOLT-4 cell (ATCC Accession No. 1582), a Namalwa cell (ATCC Accession No. CRL 1432), a Raji cell (ATCC Accession No. CCL 86), a RPMI 8226 cell (ATCC Accession No. CCL 155), a U-937 cell (ATCC Accession No. 1593), WI-28VA13 sub line 2R4 cells (ATCC Accession No. CLL 155), a CCRF-CEM cell (ATCC Accession No. CCL 119) and a 2780AD ovarian carcinoma cell (Van Der Blick et al., Cancer Res. 48:5927-5932, 1988), as well as hetero-hybridoma cells produced by fusion of human cells and cells of another species. In another embodiment, the immortalized cell line can be cell line other than a human cell line, e.g., a CHO cell line, a COS cell line. In another embodiment, the cell can be from a clonal cell strain or clonal cell line.

In a preferred embodiment, a population of cells which are capable of expressing hmGCB is provided, and at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or all of the cells produce hmGCB with at least one carbohydrate chain, preferably two, three, or four carbohydrate chains, having the specified number of mannose residues.

In a preferred embodiment, the cell is cultured in culture medium which includes at least one mannosidase inhibitor. In a preferred embodiment, the method further includes obtaining the hmGCB from the medium in which the cell is cultured.

In another aspect, the invention features an hmGCB molecule, e.g., an hmGCB molecule described herein, e.g., a human hmGCB, produced by any of the methods described herein. Preferably, the hmGCB molecule includes at least one carbohydrate chain, preferably two, three, or four carbohydrate chains, having at least four mannose residues of a precursor oligosaccharide chain.

In another aspect, the invention features an hmGCB preparation which includes a portion of hmGCB molecules which include at least one carbohydrate chain, preferably two, three, or four carbohydrate chains, having at least four mannose residues of a precursor oligosaccharide chain. Preferably, the hmGCB preparation is produced by any of the methods described herein.

In a preferred embodiment, the hmGCB is human hmGCB.

In a preferred embodiment, the hmGCB molecule can have: at least one carbohydrate chain having five mannose residues; at least one carbohydrate chain having six mannose residues; at least one carbohydrate chain having seven mannose residues; at least one carbohydrate chain having eight mannose residues; at least one carbohydrate chain having nine mannose residues.

In a preferred embodiment, the hmGCB produced (either one or more hmGCB molecules or the preparation as a whole) has at least one carbohydrate chain having a ratio of mannose residues to GlcNAc residues which is greater than 3 mannose residues to 2 GlcNAc residues, preferably the ratio of mannose to GlcNAc is 4:2, 5:2, 6:2, 7:2, 8:2, 9:2, more preferably the ratio of mannose to GlcNAc is 5:2, 8:2 or 9:2.

In a preferred embodiment, at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or all of the hmGCB of the preparation have at least one, preferably, two, three or four carbohydrate chains in which the removal of one or more mannose residues distal to the pentasaccharide core has been prevented.

In another aspect, the invention features a cell having at least one mannosidase activity inhibited and which includes a nucleic acid sequence comprising an exogenous regulatory sequence which has been introduced such that the regulatory sequence regulates the expression of an endogenous GCB coding region, wherein the cell produces GCB in which the removal of at least one mannose residue distal to the pentasaccharide core of a precursor oligosaccharide of GCB is prevented.

In a preferred embodiment, the cell produces an hmGCB preparation, e.g., a human hmGCB preparation, in which the removal of: one or more α 1,2 mannose residue(s) distal to the pentasaccharide core is prevented; an α 1,3 mannose residue distal to the pentasaccharide core is prevented; and/or an α 1,6 mannose residue distal to the pentasaccharide core is prevented. Preferably, the removal of one or more α 1,2 mannose residue(s) distal to the pentasaccharide core is prevented.

In a preferred embodiment, at least one mannosidase activity in the cell has been inhibited by contacting the cell with a substance which inhibits a mannosidase. In a preferred embodiment, the substance is a mannosidase inhibitor. The mannosidase inhibitor can be a class 1 processing mannosidase inhibitor, a class 2 processing mannosidase inhibitor or both. In a preferred embodiment, the class 1 processing mannosidase inhibitor can be one or more of: kifunensine and deoxymannojirimycin. Preferably, the class 1 processing mannosidase inhibitor is kifunensine. In a preferred embodiment, the class 2 processing mannosidase inhibitor can be one or more of: swainsonine, mannosatin, 6-deoxy-DIM, and 6-deoxy-6-fluoro-DIM. Preferably, the class 2 processing mannosidase inhibitor is swainsonine.

In a preferred embodiment, the cell carries a mutation for, e.g., a knockout for, at least one Golgi processing mannosidase. The mutation can be one which reduces the expression of the gene, reduces protein or activity levels, or alters the distribution or other post translational modifications of the mannosidase, e.g., the processing of a carbohydrate chain. The mutant can be one which reduces the level of Golgi processing mannosidase activity, e.g., one which reduces gene expression, e.g., a null mutation, e.g., a deletion, a frameshift, or an insertion. In a preferred embodiment, the mutation is a knockout in the mannosidase gene. The mutation can affect the structure (and activity of the protein), and can, e.g., be a temperature sensitive mutation. In a preferred embodiment, the cell is a mutant, e.g., a knockout, for: a class 1 processing mannosidase; a class 2 processing mannosidase; a class 1 processing mannosidase and a class 2 processing mannosidase. In a preferred embodiment, the class 1 processing mannosidase is: Golgi mannosidase IA; Golgi mannosidase IB; Golgi mannosidase IC; combinations thereof. In a preferred embodiment, the class 2 processing mannosidase is: Golgi mannosidase II.

In a preferred embodiment, the cell further includes a nucleic acid sequence, such as an antisense molecule or ribozyme, which can bind to or inactivate a cellular mannosidase nucleic acid sequence, e.g., mRNA, and inhibit expression of the protein. In a preferred embodiment, the nucleic acid sequence is: a class 1 processing mannosidase antisense molecule; a class 2 processing mannosidase antisense molecule; both a class 1 processing mannosidase

antisense molecule and a class 2 processing mannosidase antisense molecule. In a preferred embodiment, the class 1 processing mannosidase is: Golgi mannosidase IA; Golgi mannosidase IB; Golgi mannosidase IC; combinations thereof. In a preferred embodiment, the class 2 processing mannosidase is: Golgi mannosidase II.

In a preferred embodiment, the cell includes a molecule, e.g., an exogenously supplied molecule, which binds and inhibits a mannosidase. The molecule can be, e.g., a single chain antibody, an intracellular protein or a competitive or non-competitive inhibitor.

In a preferred embodiment, the hmGCB molecule produced by the cell has a ratio of mannose residues to GlcNAc residues which is greater than 3 mannose residues to 2 GlcNAc residues, preferably the ratio of mannose to GlcNAc is 4:2, 5:2, 6:2, 7:2, 8:2, 9:2, more preferably the ratio of mannose to GlcNAc is 5:2, 8:2 or 9:2.

In a preferred embodiment, the cell is unable to remove of one or more mannose residues distal to the pentasaccharide core on one, two, three or four of the carbohydrate chains of hmGCB.

In a preferred embodiment, at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or all of the hmGCB molecules produced by the cell have at least one, preferably, two, three or four carbohydrate chains in which the removal of one or more mannose residues distal to the pentasaccharide core has been prevented.

In a preferred embodiment, the regulatory sequence includes one or more of: a promoter, an enhancer, an upstream activating sequence (UAS), a scaffold-attachment region or a transcription factor-binding site. In a preferred embodiment, the regulatory sequence includes: a regulatory sequence from a metallothionein-I gene, e.g., a mouse metallothionein-I gene, a regulatory sequence from an SV-40 gene, a regulatory sequence from a cytomegalovirus gene, a regulatory sequence from a collagen gene, a regulatory sequence from an actin gene, a regulatory sequence from an immunoglobulin gene, a regulatory sequence from the HMG-CoA reductase gene, or a regulatory sequence from the EF-1 α gene.

In a preferred embodiment, the cell is: a eukaryotic cell. In a preferred embodiment, the cell is of fungal, plant or animal origin, e.g., vertebrate origin. In a preferred embodiment, the cell is: a mammalian cell, e.g., a primary or secondary mammalian cell, e.g., a fibroblast, a hematopoietic stem cell, a myoblast, a keratinocyte, an epithelial cell, an endothelial cell, a glial cell, a neural cell, a cell comprising a formed element of the blood, a muscle cell and precursors of these somatic cells; a transformed or immortalized cell line. Preferably, the cell is a human cell. Examples of immortalized human cell lines useful in the present method include, but are not limited to: a Bowes Melanoma cell (ATCC Accession No. CRL 9607), a Daudi cell (ATCC Accession No. CCL 213), a HeLa cell and a derivative of a HeLa cell (ATCC Accession Nos. CCL2, CCL2.1, and CCL 2.2), a HL-60 cell (ATCC Accession No. CCL 240), an HT-1080 cell (ATCC Accession No. CCL 121), a Jurkat cell (ATCC Accession No. TIB 152), a KB carcinoma cell (ATCC Accession No. CCL 17), a K-562 leukemia cell (ATCC Accession No. CCL 243), a MCF-7 breast cancer cell (ATCC Accession No. BTH 22), a MOLT-4 cell (ATCC Accession No. 1582), a Namalwa cell (ATCC Accession No. CRL 1432), a Raji cell (ATCC Accession No. CCL 86), a RPMI 8226 cell (ATCC Accession No. CCL 155), a U-937 cell (ATCC Accession No. 1593), WI-28VA13 sub line 2R4 cells (ATCC Accession No. CLL 155), a CCRF-CEM cell (ATCC Accession No. CCL

119) and a 2780AD ovarian carcinoma cell (Van Der Blick et al., Cancer Res. 48:5927-5932, 1988), as well as hetero-hybridoma cells produced by fusion of human cells and cells of another species. In another embodiment, the immortalized cell line can be cell line other than a human cell line, e.g., a CHO cell line, a COS cell line. In another embodiment, the cell can be from a clonal cell strain or clonal cell line.

In another aspect, the invention features a pharmaceutical composition which includes an hmGCB molecule, e.g., a human hmGCB, which includes at least one carbohydrate chain, preferably two, three, or four carbohydrate chains, having at least four mannose residues of a precursor oligosaccharide chain, in an amount suitable for treating Gaucher disease.

In a preferred embodiment, the pharmaceutical composition further includes a pharmaceutically acceptable carrier or diluent.

Another aspect of the invention features a method of treating a subject having Gaucher disease. The method includes administering to a subject having Gaucher disease an hmGCB preparation, e.g., a human hmGCB preparation, which includes at least one carbohydrate chain, preferably two, three, or four carbohydrate chains, having at least four mannose residues of a precursor oligosaccharide chain, in an amount suitable for treating Gaucher disease.

In another aspect, the invention features a method of purifying hmGCB from a sample. The method includes: providing a harvested hmGCB product; and subjecting the hmGCB product to hydrophobic charge induction chromatography (HCIC) and/or hydrophobic interaction chromatography (HIC), thereby obtaining purified hmGCB.

In a preferred embodiment, chromatography material MEP HYPERCEL® is used for HCIC. In another preferred embodiment, chromatography material MACROPREP METHYL® is used for HIC.

In another preferred embodiment, the method further includes subjecting the hmGCB product to ion exchange chromatography. The hmGCB product can be subjected to HCIC and/or HIC prior to ion exchange chromatography or the hmGCB product can be subjected to ion exchange chromatography prior to HCIC and/or HIC. Preferably, the hmGCB product is subjected to more than one ion exchange chromatography step. The ion exchange chromatography can be: anion exchange chromatography, cation exchange chromatography or both.

In a preferred embodiment, anion exchange chromatography is performed using one or more of the following chromatography materials: Q SEPHAROSE FAST FLOW®, MACROPREP HIGH Q SUPPORT®, DEAE SEPHAROSE FAST FLOW®, AND MACRO-PREP DEAE®. In a preferred embodiment, cation exchange chromatography is performed using one or more of: SP Sepharose Fast Flow®, Source 30S®, CM Sepharose Fast Flow®, Macro-Prep CM Support®, and Macro-Prep High S Support®.

In a preferred embodiment, the method further includes subjecting the hmGCB product to size exclusion chromatography. Preferably, the size exclusion chromatography is performed using one or more of the following chromatography materials: SUPERDEX 200®, SEPHACRYL S-200 HR® AND BIO-GEL A 1.5M®.

In another aspect, the invention features a method of purifying hmGCB. The method includes: providing a harvested hmGCB product; subjecting the hmGCB product to hydrophobic charge induction chromatography (HCIC) and/or hydrophobic interaction chromatography (HIC); and subjecting the hmGCB product to one or more of anion

exchange chromatography, cation exchange chromatography, and size exclusion chromatography, to thereby obtain purified hmGCB.

In a preferred embodiment, chromatography material MEP HYPERCEL® is used for HCIC. In another preferred embodiment, chromatography material MACROPREP METHYL® is used for HIC.

In a preferred embodiment, the method includes using anion exchange chromatography. Preferably, anion exchange chromatography is performed using one or more of the following chromatography materials: Q SEPHAROSE FAST FLOW®, MACROPREP HIGH Q SUPPORT®, DEAE SEPHAROSE FAST FLOW®, AND MACRO-PREP DEAE®.

In a preferred embodiment, the method includes using cation exchange chromatography. Preferably, cation exchange chromatography is performed using one or more of the following chromatography materials: SP SEPHAROSE FAST FLOW®, SOURCE 30S®, CM SEPHAROSE FAST FLOW®, MACRO-PREP CM SUPPORT®, AND MACRO-PREP HIGH S SUPPORT®.

In a preferred embodiment, the method includes using size exclusion chromatography. Preferably, the size exclusion chromatography is performed using one or more of the following chromatography materials: SUPERDEX 200®, SEPHACRYL S-200 HR® AND BIO-GEL A 1.5M®.

In a preferred embodiment, the hmGCB is subjected to (in any order): anion exchange chromatography and cation exchange chromatography; anion exchange chromatography and size exclusion chromatography; cation exchange chromatography and size exclusion chromatography; anion exchange chromatography, cation exchange chromatography and size exclusion chromatography. Preferably, the hmGCB is subjected to all three of these chromatography steps in the following order: anion exchange chromatography, cation exchange chromatography and size exclusion chromatography.

In another aspect, the invention features a method of purifying hmGCB. The method includes: providing a harvested hmGCB product; subjecting the hmGCB product to hydrophobic charge induction chromatography (HCIC) and/or hydrophobic interaction chromatography (HIC); subjecting the HCIC and/or HIC purified hmGCB product to anion exchange chromatography; subjecting the anion exchange purified hmGCB to cation exchange chromatography; and, subjecting the cation exchange purified hmGCB to size exclusion chromatography, to thereby obtain purified hmGCB.

In a preferred embodiment, chromatography material MEP HYPERCEL® is used for HCIC. In another preferred embodiment, chromatography material MACROPREP METHYL® is used for HIC.

In a preferred embodiment, anion exchange chromatography is performed using one or more of the following chromatography materials: Q Sepharose Fast Flow®, MacroPrep High Q Support®, DEAE Sepharose Fast Flow®, and Macro-Prep DEAE®.

In a preferred embodiment, cation exchange chromatography is performed using one or more of the following chromatography materials: SP SEPHAROSE FAST FLOW®, SOURCE 30S®, CM SEPHAROSE FAST FLOW®, MACRO-PREP CM SUPPORT®, AND MACRO-PREP HIGH S SUPPORT®.

In a preferred embodiment, size exclusion chromatography is performed using one or more of the following chromatography materials: SUPERDEX 200®, SEPHACRYL S-200 HR® AND BIO-GEL A 1.5M®.

The term "high mannose glucocerebrosidase (hmGCB)" as used herein refers to glucocerebrosidase having at least one carbohydrate chain having four or more mannose residues from a precursor oligosaccharide. Preferably, the hmGCB has five, six, seven, eight or nine mannose residues from the precursor oligosaccharide chain. Most preferably, the hmGCB has five, eight or nine mannose residues from the precursor oligosaccharide chain.

The term "hmGCB preparation" refers to two or more hmGCB molecules.

The term "primary cell" includes cells present in a suspension of cells isolated from a vertebrate tissue source (prior to their being plated i.e., attached to a tissue culture substrate such as a dish or flask), cells present in an explant derived from tissue, both of the previous types of cells plated for the first time, and cell suspensions derived from these plated cells. The term secondary cell or cell strain refers to cells at all subsequent steps in culturing. That is, the first time a plated primary cell is removed from the culture substrate and replated (passaged), it is referred to herein as a secondary cell, as are all cells in subsequent passages. Secondary cells are cell strains which consist of secondary cells which have been passaged one or more times. A cell strain consists of secondary cells that: 1) have been passaged one or more times; 2) exhibit a finite number of mean population doublings in culture; 3) exhibit the properties of contact-inhibited, anchorage dependent growth (anchorage-dependence does not apply to cells that are propagated in suspension culture); and 4) are not immortalized. A "clonal cell strain" is defined as a cell strain that is derived from a single founder cell. A "heterogenous cell strain" is defined as a cell strain that is derived from two or more founder cells.

"Immortalized cells", as used herein, are cell lines (as opposed to cell strains with the designation "strain" reserved for primary and secondary cells), a critical feature of which is that they exhibit an apparently unlimited lifespan in culture.

The term "transfected cell" refers to a cell into which an exogenous synthetic nucleic acid sequence, e.g., a sequence which encodes a protein, is introduced. Once in the cell, the synthetic nucleic acid sequence can integrate into the recipient's cells chromosomal DNA or can exist episomally. Standard transfection methods can be used to introduce the synthetic nucleic acid sequence into a cell, e.g., transfection mediated by liposome, polybrene, DEAE dextran-mediated transfection, electroporation, calcium phosphate precipitation or microinjection. The term "transfection" does not include delivery of DNA or RNA into a cell by a virus.

The term "infected cell" or "transduced cell" refers to a cell into which an exogenous synthetic nucleic acid sequence, e.g., a sequence which encodes a protein, is introduced by a virus. Viruses known to be useful for gene transfer include an adenovirus, an adeno-associated virus, a herpes virus, a mumps virus, a poliovirus, a retrovirus, a Sindbis virus, a lentivirus and a vaccinia virus such as a canary pox virus.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

BRIEF DESCRIPTION OF THE DRAWING

FIG. 1 is a diagram showing the trimming of N-linked glycans as it occurs in the endoplasmic reticulum, the intermediate compartment and in the Golgi apparatus. The enzymes are numbered as follows: (1) α -glucosidase I; (2) α -glucosidase II; (3) ER mannosidase I; (4) ER mannosidase

II; (5) ER glucosyl transferase; (6) endomannosidase; (7) Golgi mannosidase IA, IB and IC; (8) GlcNAc transferase I; (9) Golgi mannosidase II. Δ : Glucose; \square : GlcNAc; \bullet : Mannose. Enzymes (3) and (7) are inhibited by kifunensine; enzyme (9) is inhibited by swainsonine.

DETAILED DESCRIPTION OF THE INVENTION

The invention is based, in part, on the discovery that inhibition of the removal of one or more mannose residues distal from the pentasaccharide core of a precursor oligosaccharide chain of glucocerebrosidase (GCB), results in high mannose glucocerebrosidase (hmGCB) that is efficiently targeted to mannose receptors. The removal of a mannose residue from the pentasaccharide core of a precursor oligosaccharide chain can be prevented by inhibiting or reducing the activity of one or more mannosidase enzymes, e.g., one or more class I processing mannosidase(s) and/or class 2 processing mannosidase(s). By preventing or inhibiting the removal of one or more mannose residues, hmGCB having at least one carbohydrate chain with four or more mannose residues from the precursor oligosaccharide chain can be obtained.

Gaucher disease is caused by a deficiency of GCB. GCB is required for degradation of glycosphingolipid glucocerebroside. In the absence of GCB, the glucocerebroside accumulates primarily in phagocytic cells, e.g., macrophages, and, ultimately, builds up in the liver, spleen and bone marrow.

Macrophages have mannose receptors. These receptors play a role in receptor-mediated endocytosis by these cells. hmGCB efficiently targets the mannose receptors on macrophages and improves the uptake of GCB (in the form of hmGCB) into these cells. By directing GCB (in the form of hmGCB) to the cells in which glucocerebroside accumulates, hmGCB can be used to hydrolyze glucocerebroside in the macrophages, thereby reducing the subsequent accumulation of this glycolipid in the liver, spleen and bone marrow of patients having Gaucher disease.

Glucocerebrosidase

Nucleotide sequence information is available for genes encoding glucocerebrosidase from various species. (See Horowitz et al. (1989) *Genomics* 4(1):87-96, disclosing the gene sequence (SEQ ID NO: 1) and amino acid sequence (SEQ ID NO:2) of human glucocerebrosidase; Beutler et al. (1992) *Genomics* 12(4):795-800).

Mature human GCB has five potential N-linked glycosylation sites at Asn-19, Asn-59, Asn-146, Asn-270, and Asn-462. Glycosylation occurs at four of the five sites in human tissue derived GCB (Erickson et al. (1985) *J. Biol. Chem.* 260:14319-14324). Studies employing site-directed mutagenesis have demonstrated that the site at Asn-462 is never occupied (Berg-Fussman et al. (1993) *J. Biol. Chem.* 268:14861-14866). Approximately 20% of the released glycan chains from human placental GCB were shown to be of the high mannose type containing up to seven mannose residues, whereas the majority of the glycan chains were of the complex type with sialylated biantennary and triantennary structures. (Takasaki et al. (1984) *J. Biol. Chem.* 259:10112-10117)

The first event in GCB N-glycosylation is the co-translational transfer in the lumen of the endoplasmic reticulum (ER) of Glc₃Man₅GlcNAc₂ from oligosaccharide-PP-dolichol to nascent peptide. The presence of the three glucose residues on the donor oligosaccharide allows for efficient

transfer to an acceptor asparagine by oligosaccharyl transferase. Following N-glycosylation, the glucose residues are rapidly removed from GCB during the folding process by ER glucosidases I and II. Two different ER mannosidases are each capable of hydrolyzing a single mannose residue from $\text{Man}_5\text{GlcNAc}_2$ to form two different isomers of $\text{Man}_4\text{GlcNAc}_2$ (see FIG. 1). Accessible glycans are then further processed in the Golgi to $\text{Man}_3\text{GlcNAc}_2$ by the removal of up to four $\alpha 1,2$ -linked mannose residues by Golgi mannosidase I. There are at least three different human genes encoding related Golgi mannosidase I isoforms (IA, IB, and IC) with slightly different substrate specificities and tissue expression but all are capable of trimming four mannose residues from $\text{Man}_5\text{GlcNAc}_2$ glycans to form $\text{Man}_3\text{GlcNAc}_2$ (Tremblay et al. (Jul. 27, 2000) *J. Biol. Chem.* [pub ahead of print]). They are located on chromosomes 6q22, 1p13, and 1p35-36 and their cDNA sequences are obtainable from GenBank as X74837, AF027156, and AF261655, respectively.

The final stage of processing that commits a glycan to the biosynthetic pathway for complex glycans requires the initial conversion of $\text{Man}_3\text{GlcNAc}_2$ to $\text{GlcNAcMan}_3\text{GlcNAc}_2$ by the action of GlcNAc transferase I, after which Golgi mannosidase II can catalyze the removal of two further mannose residues to yield $\text{GlcNAcMan}_2\text{GlcNAc}_2$. This is the substrate for glycan elongation by glycosyl transferases located in the trans Golgi and the trans Golgi network to form complex type chains.

If the high mannose chains transferred to GCB in the initial N-glycosylation step can be prevented from being processed to complex chains in the Golgi, then GCB with high mannose chains (hmGCB) will effectively target the mannose receptors on reticuloendothelial cells.

Cells

Primary and secondary cells to be transfected or infected can be obtained from a variety of tissues and include cell types which can be maintained and propagated in culture. For example, primary and secondary cells which can be transfected or infected include fibroblasts, keratinocytes, epithelial cells (e.g., mammary epithelial cells, intestinal epithelial cells), endothelial cells, glial cells, neural cells, formed elements of the blood (e.g., lymphocytes, bone marrow cells), muscle cells and precursors of these somatic cell types. Primary cells are preferably obtained from the individual to whom the transfected or infected primary or secondary cells are administered (i.e., an autologous cell). However, primary cells may be obtained from a donor (other than the recipient) of the same species (i.e., an allogeneic cell) or another species (i.e., a xenogeneic cell) (e.g., mouse, rat, rabbit, cat, dog, pig, cow, bird, sheep, goat, horse, monkey, baboon).

Primary or secondary cells of vertebrate, particularly mammalian, origin can be transfected or infected with an exogenous DNA sequence, e.g., an exogenous DNA sequence encoding a therapeutic protein, and produce an encoded therapeutic protein stably and reproducibly, both in vitro and in vivo, over extended periods of time. In addition, the transfected or infected primary and secondary cells can express the encoded product in vivo at physiologically relevant levels, cells can be recovered after implantation and, upon reculturing, to grow and display their preimplantation properties. Cells can be modified to reduce cell surface histo compatibility complex or foreign carbohydrate moieties to reduce immunogenicity, e.g., a universal donor cell.

Alternatively, primary or secondary cells of vertebrate, particularly mammalian, origin can be transfected or infected with an exogenous DNA sequence which includes a regulatory sequence. Examples of such regulatory sequences include one or more of: a promoter, an UAS, a scaffold attachment region or a transcription binding site. The targeting event can result in the insertion of the regulatory sequence of the DNA sequence, placing a targeted endogenous gene under their control (for example, by insertion of either a promoter or an enhancer, or both, upstream of the endogenous gene or regulatory region). Optionally, the targeting event can simultaneously result in the deletion of an endogenous regulatory sequence, such as the deletion of a tissue-specific negative regulatory sequence, of a gene. The targeting event can replace an existing regulatory sequence; for example, a tissue-specific enhancer can be replaced by an enhancer that has broader or different cell-type specificity than the endogenous elements, or displays a pattern of regulation or induction that is different from the corresponding nontransfected or noninfected cell. In this regard, the endogenous sequences are deleted and new sequences are added. Alternatively, the endogenous regulatory sequences are not removed or replaced but are disrupted or disabled by the targeting event, such as by targeting the exogenous sequences within the endogenous regulatory elements. Introduction of a regulatory sequence by homologous recombination can result in primary or secondary cells expressing a therapeutic protein which it does not normally express. In addition, targeted introduction of a regulatory sequence can be used for cells which make or contain the therapeutic protein but in lower quantities than normal (in quantities less than the physiologically normal lower level) or in defective form, and for cells which make the therapeutic protein at physiologically normal levels, but are to be augmented or enhanced in their content or production. Methods of activating an endogenous coding sequence are described in U.S. Pat. Nos. 5,641,670, 5,733,761 and 5,968,502, the contents of which are incorporated herein by reference.

The transfected or infected primary or secondary cells may also include a DNA sequence encoding a selectable marker which confers a selectable phenotype upon them, facilitating their identification and isolation. Methods for producing transfected primary or secondary cells which stably express the DNA sequence, clonal cell strains and heterogenous cell strains of such transfected cells, methods of producing the clonal and heterogenous cell strains, are known and described, for example, in U.S. Pat. Nos. 5,641,670, 5,733,761 and 5,968,502, the contents of which are incorporated herein by reference.

Transfected primary or secondary cells, can be made by electroporation. Electroporation is carried out at appropriate voltage and capacitance (and corresponding time constant) to result in entry of the DNA construct(s) into the primary or secondary cells. Electroporation can be carried out over a wide range of voltages (e.g., 50 to 2000 volts) and corresponding capacitance. Total DNA of approximately 0.1 to 500 μg is generally used.

Alternatively, known methods such as calcium phosphate precipitation, microinjection, modified calcium phosphate precipitation and polybrene precipitation, liposome fusion and receptor-mediated gene delivery can be used to transfect cells.

Processing of Glucocerebrosidase

Oligosaccharide assembly in cells which have not been treated to prevent removal of mannose residues usually proceeds as discussed below:

The oligosaccharide chains of GCB are attached to the polypeptide backbone by N-glycosidic linkages. N-linked glycans have an amide bond that connects the anomeric carbon (C-1) of a reducing-terminal N-acetylglucosamine (GlcNAc) residue of the oligosaccharide and a nitrogen of an asparagine (Asn) residue of the polypeptide.

Initiation of N-linked oligosaccharide assembly does not occur directly on the Asn residues of the GCB protein, but rather involves preassembly of a lipid-linked 14 sugar precursor oligosaccharide which is then transferred to the protein in the ER during or very soon after its translation from mRNA. A "precursor oligosaccharide" as used herein refers to the oligosaccharide chain involved in the initial steps in biosynthesis of carbohydrate chains. A "precursor oligosaccharide" can be an oligosaccharide structure which includes at least the following sugars: $\text{Man}_2\text{GlcNAc}_2$, for example, a precursor oligosaccharide can have the following structure: $\text{Glc}_2\text{Man}_3\text{GlcNAc}_2$, as shown in FIG. 1. The precursor oligosaccharide is synthesized while attached via a pyrophosphate bridge to a polyisoprenoid carrier lipid, a dolichol. This assembly involves at least six distinct membrane bound glycosyltransferases. Some of these enzymes transfer monosaccharides from nucleotide sugars, while others utilize dolichol-linked monosaccharides as sugar donors. After assembly of the lipid-linked precursor is complete, another membrane-bound enzyme transfers it to sterically accessible Asn residues which occur as part of the sequence -Asn-X-Ser/Thr.

Glycosylated Asn residues of newly-synthesized GCB transiently carry $\text{Glc}_2\text{Man}_3\text{GlcNAc}_2$, also referred to herein as an "unprocessed carbohydrate chain".

The processing of N-linked oligosaccharides is accomplished by the sequential action of a number of membrane-bound enzymes and begins immediately after transfer of the precursor oligosaccharide $\text{Glc}_2\text{Man}_3\text{GlcNAc}_2$ to the protein. The terms "processing", "trimming" and "modifying" are used interchangeably herein.

N-linked oligosaccharide processing can be divided into three stages: removal of the three glucose residues, removal of a variable number of mannose residues, and addition of various sugar residues to the resulting trimmed core.

The removal of the glucose residues in the first stage of processing involves removal of all three glucose residues to generate N-linked $\text{Man}_3\text{GlcNAc}_2$. This structure is also referred to herein as: $\text{Man}\alpha 1-2\text{Man}\alpha 1-2\text{Man}\alpha 1-3[\text{Man}\alpha 1-2\text{Man}\alpha 1-3(\text{Man}\alpha 1-2\text{Man}\alpha 1-6)\text{Man}\alpha 1-6]\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}$ (See FIG. 1, structure 9'). Processing normally continues to the second stage with removal of mannose residues.

Four of the mannose residues of the $\text{Man}_3\text{GlcNAc}_2$ moiety are bound by $\alpha 1,2$ linkages. Up to four of these $\alpha 1,2$ -linked mannose residues can be removed by mannosidase IA, IB and IC to generate N-linked $\text{Man}_{3-6}\text{GlcNAc}_2$.

Protein-linked $\text{Man}_3\text{GlcNAc}_2$ can then serve as a substrate for GlcNAc transferase I, which transfers a $\beta 1,2$ -linked GlcNAc residue from UDP-GlcNAc to the core $\alpha 1,3$ -linked mannose residue to form $\text{GlcNAcMan}_3\text{GlcNAc}_2$. Mannosidase II can then complete the trimming phase of the processing pathway by removing two mannose residues to generate a protein-linked oligosaccharide which contains within it a $\text{Man}_2\text{GlcNAc}_2$, the "pentasaccharide core". The structure $\text{GlcNAcMan}_2\text{GlcNAc}_2$ is then a substrate for GlcNAc transferase II, which can transfer a $\beta 1,2$ -linked GlcNAc residue to the $\alpha 1,6$ -linked mannose residue.

After the trimming phase, monosaccharides are sequentially added to the growing oligosaccharide chain by a series of membrane-bound Golgi glycosyltransferases, each of

which is highly specific with respect to the acceptor oligosaccharide, the donor sugar, and the type of linkage formed between the sugars. These can include distinct GlcNAc transferases (producing $\beta 1,2$; $\beta 1,4$; or $\beta 1,6$ linkages); galactosyltransferases (producing $\beta 1,4$; $\beta 1,3$; and $\alpha 1,3$ linkages); sialyltransferases (one producing $\alpha 2,3$ and another, $\alpha 2,6$ linkages); fucosyltransferases (producing $\alpha 1,2$; $\alpha 1,3$; $\alpha 1,4$ or $\alpha 1,6$ linkages); and a growing list of other enzymes responsible for a variety of unusual linkages. The cooperative action of these glycosyltransferases produces a diverse family of structures collectively referred to as "complex" oligosaccharides. These may contain two, three or four outer branches ("antennae") attached to the invariant core pentasaccharide, $\text{Man}_2\text{GlcNAc}_2$. These structures are referred to in terms of the number of their outer branches: biantennary (two branches), triantennary (three branches) or tetraantennary (four branches). The size of these complex glycans can vary.

Processing of High Mannose Glucocerebrosidase

hmGCB can be produced by reducing or preventing cellular carbohydrate modification (i.e., processing) of GCB. Carbohydrate modification can be prevented by allowing production of GCB under conditions which prevent the removal of at least one mannose residue distal to the pentasaccharide core of a precursor oligosaccharide chain of GCB. For example, one or more of the "trimming" stages during the removal of mannose residues from a precursor oligosaccharide can be prevented.

Cellular mannosidases fall into two broad classes: class 1 processing enzymes, which include ER mannosidase I, Golgi mannosidase IA, IB and IC and which hydrolyze $\alpha 1,2$ -linked mannose residues, and require Ca^{2+} for activity; and class 2 processing enzymes, which include ER mannosidase II, Golgi mannosidase II, cytosolic α -mannosidase, and lysosomal α -mannosidase and which have a broader substrate specificity and do not require Ca^{2+} for activity.

The trimming of mannose residues from the precursor oligosaccharide involves at least the following mannosidase enzymes: Golgi mannosidase IA, IB and IC, and Golgi mannosidase II. By inhibiting one or more of these mannosidases during N-linked oligosaccharide assembly in a cell, GCB can be produced which has at least one carbohydrate chain with one or more mannose residues in addition to the pentasaccharide core. For example, inhibition of both ER mannosidase I and Golgi mannosidase I can produce hmGCB with at least one carbohydrate chain (and preferably all chains) having at least eight mannose residues from the precursor oligosaccharide; inhibition of Golgi mannosidase II can produce hmGCB with at least one carbohydrate chain (and preferably all chains) having at least five mannose residues from the precursor oligosaccharide.

Trimming by a mannosidase can be inhibited, for example, by contacting the cell with a substance which prevents the removal of one or more mannose residues from a precursor oligosaccharide of GCB or by producing GCB in a cell which does not produce or produces at deficient levels at least one mannosidase, or in a cell which produces a mutated and/or inactive mannosidase. For example, the cell can be a knockout for at least one mannosidase, can express at least one antisense mannosidase molecule or can be dominant negative for at least one mannosidase.

Substances which Prevent Removal of Mannose Residues

A substance which prevents the removal of one or more mannose residues from a precursor oligosaccharide of GCB can be used to produce an hmGCB preparation. For example, a cell which expresses GCB can be contacted with

a substance which prevents the removal of one or more α 1,2 mannose residues of a precursor oligosaccharide of GCB, and/or removal of an α 1,3 mannose residue of a precursor oligosaccharide of GCB, and/or removal of an α 1,6 mannose residue of a precursor oligosaccharide of GCB. Preferably, the substance is a mannosidase inhibitor, e.g., a class 1 processing mannosidase inhibitor or a class 2 processing mannosidase inhibitor.

Cellular mannosidases fall into two broad classes on the basis of protein sequence homologies (Moremen et al. (1994) *Glycobiology* 4:113-125). These two classes are mechanistically different. Class 1 enzymes, which include ER mannosidase I and Golgi mannosidase I isoforms, have a mass of about 63-73 kDa, hydrolyze α 1,2-linked mannose residues and require Ca^{2+} for activity. Class 1 processing mannosidases can be blocked, for example, by treatment with a substrate mimic, e.g., a pyranose analog of mannose. For example, class 1 processing mannosidases can be blocked by treatment with one or more of the following enzymatic inhibitors: kifunensine, deoxymannojirimycin, or a combination thereof. Class 2 enzymes, which include ER mannosidase I, Golgi mannosidase II, cytosolic α -mannosidase, and lysosomal α -mannosidase, have a greater mass of about 107-136 kDa, do not require Ca^{2+} for activity and have a broader substrate specificity. Class 2 processing mannosidases can be blocked, for example, by treatment with furanose transition state analogues of the mannosylation (Daniels et al. (1994) *GlycoBiol.* 4:551-566). For example, class 2 processing mannosidases can be blocked by treatment with one or more of the following inhibitors: swainsonine, 6-deoxy-DIM, 6-deoxy-6-fluoro-DIM, mannosidase A, or combinations thereof.

Kifunensine can be used as an inhibitor of the endoplasmic reticulum mannosidase I and/or Golgi mannosidase IA and/or IB and/or IC; deoxymannojirimycin can be used as an inhibitor of ER mannosidase I, ER mannosidase II and/or of Golgi mannosidase IA and/or IB and/or IC; swainsonine can be used as an inhibitor of Golgi mannosidase II; and mannosidase A can be used as an inhibitor of Golgi mannosidase II.

Use of a mannosidase inhibitor can inhibit the processing of a carbohydrate chain of GCB past a certain stage of mannose residue trimming during oligosaccharide assembly. For example, contacting a cell with kifunensine can inhibit trimming of any, or one, two, three, or four of the mannose residues of a precursor oligosaccharide.

Processing α -mannosidases can be blocked by treatment of cells with one or more of the following enzyme inhibitors:

Kifunensine, an inhibitor of the endoplasmic reticulum I and Golgi mannosidase I enzymes (Weng and Spiro (1993) *J. Biol. Chem.* 268:25656-25663; Elbein et al. (1990) *J. Biol. Chem.* 265:15599-15605).

Swainsonine, an inhibitor of the Golgi mannosidase II enzyme (Tulsiani et al. (1982) *J. Biol. Chem.* 257: 7936-7939).

Deoxymannojirimycin, an inhibitor of both endoplasmic reticulum mannosidases I and II and of Golgi mannosidase I (Weng and Spiro (1993) *J. Biol. Chem.* 268: 25656-25663; Tremblay and Herscovics (2000) *J. Biol. Chem.* July 27; [pub ahead of print]).

DIM (1,4-dideoxy-1,4-imino-D-mannitol), an inhibitor of Golgi mannosidase II (Palamarzyk et al. (1985) *Arch. Biochem. Biophys.* 243:35-45).

6-Deoxy-DIM and 6-deoxy-6-fluoro-DIM, inhibitors of Golgi mannosidase II (Winchester et al. (1993) *Biochem. J.* 290:743-749).

Mannostatin A, an inhibitor of Golgi mannosidase II (Tropea et al. (1990) *Biochemistry* 29:10062-10069).

Various mannosidase inhibitors can be selected by their ability to penetrate particular cell types as well as by the inhibitory potency of the mannosidase inhibitor. For example, swainsonine is rapidly internalized by cultured fibroblasts in a time- and concentration-dependent manner. Swainsonine is also a potent inhibitor of a class 2 mannosidase, e.g., Golgi mannosidase II. Thus, swainsonine can be used to produce hmGCB in cultured fibroblasts, e.g., hmGCB having at least one carbohydrate chain which has at least four or five mannose residues of the precursor oligosaccharide. In addition, kifunensine is readily taken up by cultured fibroblasts and is a potent inhibitor of class 1 mannosidases, e.g., ER mannosidase I and Golgi mannosidase I. Thus, kifunensine can be used to produce hmGCB in cultured fibroblasts, e.g., hmGCB having at least one carbohydrate chain which has at least four, five, six, seven, eight or nine mannose residues of the precursor oligosaccharide.

Preferably, the mannosidase inhibitor is present at a concentration of 0.025 to 20.0 $\mu\text{g/ml}$, 0.05 to 10 $\mu\text{g/ml}$, 0.05 to 5 $\mu\text{g/ml}$, preferably between about 0.1 to 2.0 $\mu\text{g/ml}$. For example, a class 1 processing mannosidase inhibitor can be present at a concentration between about 0.025 to 20.0 $\mu\text{g/ml}$, 0.05 to 10 $\mu\text{g/ml}$, 0.05 to 5 $\mu\text{g/ml}$, preferably between about 0.1 to 2.0 $\mu\text{g/ml}$; a class 2 processing mannosidase inhibitor can be present at a concentration between about 0.025 to 20.0 $\mu\text{g/ml}$, 0.05 to 10 $\mu\text{g/ml}$, 0.05 to 5 $\mu\text{g/ml}$, preferably between about 0.1 to 2.0 $\mu\text{g/ml}$; each of the class 1 processing and class 2 processing mannosidase inhibitors can be present at a concentration between about 0.025 to 20.0 $\mu\text{g/ml}$, 0.05 to 10 $\mu\text{g/ml}$, 0.05 to 5 $\mu\text{g/ml}$, preferably between about 0.1 to 2.0 $\mu\text{g/ml}$; or the total concentration of the class 1 processing and class 2 processing mannosidase inhibitors present can be between about 0.025 to 40.0 $\mu\text{g/ml}$, 0.05 to 20 $\mu\text{g/ml}$, 0.05 to 10 $\mu\text{g/ml}$, preferably between about 0.1 to 5.0 $\mu\text{g/ml}$.

The cell can be contacted with a mannosidase inhibitor by, for example, culturing the cell on medium which includes at least one mannosidase inhibitor.

Mannosidase Mutant Cell

Mannosidase Knockout Cell

Permanent or regulated inactivation of mannosidase gene expression can be achieved by targeting to a mannosidase locus with a transfected plasmid DNA construct or a synthetic oligonucleotide. The plasmid construct or oligonucleotide can be designed to several forms. These include the following: 1) insertion of selectable marker genes or other sequences within an exon of a mannosidase gene; 2) insertion of exogenous sequences in regulatory regions of non-coding sequence; 3) deletion or replacement of regulatory and/or coding sequences; and, 4) alteration of a protein coding sequence by site specific mutagenesis.

In the case of insertion of a selectable marker gene into coding sequence, it is possible to create an in-frame fusion of an endogenous mannosidase exon with the mannosidase exon engineered to contain, for example, a selectable marker gene. In this way following successful targeting, the endogenous mannosidase gene expresses a fusion mRNA (mannosidase sequence plus selectable marker sequence). Moreover, the fusion mRNA would be unable to produce a functional mannosidase translation product.

In the case of insertion of DNA sequences into regulatory regions, the transcription of a mannosidase gene can be silenced by disrupting the endogenous promoter region or any other regions in the 5' untranslated region (5' UTR) that is needed for transcription. Such regions include, for

example, translational control regions and splice donors of introns. Secondly, a new regulatory sequence can be inserted upstream of the mannosidase gene that would render the mannosidase gene subject to the control of extracellular factors. It would thus be possible to down-regulate or extinguish mannosidase gene expression as desired for optimal hmGCB production. Moreover, a sequence which includes a selectable marker and a promoter can be used to disrupt expression of the endogenous sequence. Finally, all or part of the endogenous mannosidase gene could be deleted by appropriate design of targeting substrates.

In order to create a cell which includes a knockout of at least one chromosomal copy of the human Golgi mannosidase IA, IB or IC gene, the genomic DNA comprising at least the 5' portion of the gene (including regulatory sequences, 5' UTR, coding sequence) is isolated. For example, the GenBank sequence, Accession No.: NM005907 (human), can be used to generate a probe for Golgi mannosidase IA or Accession Nos.: AAF97058 can be used to generate a probe for Golgi mannosidase IB or IC using polymerase chain reaction (PCR). Oligonucleotides for PCR can be designated based upon the GenBank sequence. The resulting probe can hybridize to the single copy Golgi mannosidase IA, IB or IC gene. This probe can then be used to screen a commercially available recombinant phage library (e.g., a library made from human genomic DNA) to isolate a clone comprising all or part of the mannosidase I structural genes. Once a recombinant clone comprising a mannosidase regulatory and/or coding sequence is isolated, specific targeting plasmids designed to achieve the inactivation of mannosidase gene expression can then be constructed. Inactivation of mannosidase activity results from the insertion of exogenous DNA into regulatory or coding sequences to disrupt the translational reading frame. Inactivation of the enzyme can also be the result of disruption of mRNA transcription or mRNA processing, or by deletion of endogenous mannosidase regulatory or coding sequences.

The nucleic acid sequence of other class 1 and class 2 processing mannosidase are also available, for example, in GenBank. Using the methods described above for Golgi mannosidase IA, IB or IC, a knockout cell for other class 1 and/or class 2 processing mannosidases can be produced.

A mannosidase knockout cell can be used, for example, in gene therapy. A knockout cell can be administered to a subject, e.g., a subject having Gaucher disease, such that the cell produces hmGCB in vivo.

Antisense Mannosidase Nucleic Acid Sequences

Nucleic acid molecules which are antisense to a nucleotide encoding a mannosidase, e.g., a class 1 processing or class 2 processing mannosidase, can be used as an inactivating agent which inhibits expression of a mannosidase. For example, Golgi mannosidase IA, Golgi mannosidase IB, Golgi mannosidase IC, and/or Golgi mannosidase II expression can be inhibited by an antisense nucleic acid molecule. An "antisense" nucleic acid includes a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a mannosidase, e.g., complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. Accordingly, an antisense nucleic acid can form hydrogen bonds with a sense nucleic acid. The antisense nucleic acid can be complementary to an entire mannosidase coding strand, or to only a portion thereof. For example, an antisense nucleic acid molecule which antisense to the "coding region" of the coding strand of a nucleotide sequence encoding a mannosidase can be used.

As the coding strand sequences encoding various mannosidases are disclosed in, for example, Bause (1993) *Eur. J. Biochem.* 217(2):535-540; Gonzalez et al. (1999) *J. Biol. Chem.* 274(30):21375-21386; Misago et al. (1995) *Proc. Natl. Acad. Sci. USA* 92(25): 11766-11770; Tremblay et al. (1998) *Glycobiology* 8(6):585-595; Tremblay et al. (2000) *J. Biol. Chem.* July 27:[epub ahead of print], antisense nucleic acids can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule can comprise sequence complementary to the entire coding region of a mannosidase mRNA, but more preferably is an oligonucleotide which is complementary to only a portion of the coding or noncoding region of a mannosidase mRNA. For example, the antisense oligonucleotide can comprise sequence complementary to the region surrounding the translation start site of a mannosidase mRNA. An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, or more nucleotides in length. An antisense nucleic acid can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (e.g., an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, e.g., phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl)uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N-6-isopentenyladenine, uracil-5-oxyacetic acid (v), ybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methyl-ester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (i.e., RNA transcribed from the inserted nucleic acid will be of an antisense orientation relative to a target nucleic acid of interest).

Purification of hmGCB

The term "purified" hmGCB, as used herein, refers to hmGCB that is substantially free of cellular material when produced by a cell which expresses GCB. The language "substantially free of cellular material" includes preparations of hmGCB in which the protein is separated from cellular components of the cells in which it is produced. In one embodiment, the language "substantially free of cellular material" includes preparations of hmGCB having less than about 30% (by dry weight) of non-GCB protein (also referred to herein as a "protein impurity" or "contaminating protein"), more preferably less than about 20% of non-GCB protein, still more preferably less than about 10% of non-GCB protein, and most preferably less than about 5%.

non-GCB protein. When the hmGCB is obtained (i.e., harvested) from culture media, it is also preferably substantially free of a component of the culture medium, i.e., components of the culture medium represent less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the dry weight of the protein preparation.

Various methods can be used to harvest hmGCB from culture media. The term "harvested hmGCB" as used herein refers to hmGCB obtained from culture media or from a cell. For example, one of the following alternatives can be used to prepare the harvested hmGCB prior to a purification procedure. These can include: 1) filtering the fresh harvest; 2) filtering the fresh harvest and freezing, e.g., at about -20° C. to -80° C., the filtered product until ready for processing (at which time it can be thawed and, optionally, filtered); 3) filtering the fresh harvest, concentrating filtered product (e.g., by about 8 to 10 fold), and then, optionally, filtering again; 4) filtering the fresh harvest, concentrating filtered product (e.g., by about 8 to 10 fold), optionally, filtering again, and then freezing, e.g., at about -20° C. to -80° C., until ready for processing (at which time it can be thawed and, optionally, filtered). Variations of these alternatives can also be performed. For example, when the harvested product or concentrated harvested product is frozen, different harvests can be pooled after thawing and filtered. In addition, for harvested or concentrated harvested product, the product can be held at a cooling temperature, e.g., about 2° C. to 8° C., for short periods of time, e.g., about 1 to 3 days, preferably 1 day, prior to purification. The harvested product held at the cooling temperature can be pooled prior to purification.

When a concentration of harvest is performed, an ultra-filtration membrane with a 5,000 to 50,000 mw cutoff, preferably a 10,000 to 30,000 mw cutoff, can be employed. Filter clarification will typically employ a 1.2 µm/0.5 µm prefilter, followed by a 0.2 µm final filter.

HmGCB can be purified by the following purification techniques. For example, hydrophobic charge induction chromatography (HCIC) can be used to purify the hmGCB preparation. Alternatively, hydrophobic interaction chromatography (HIC) can be used to purify the hmGCB preparation. Both HCIC and HIC are described below.

HCIC or HIC can be used alone or in combination with one or more ion exchange steps. Ion exchange steps that can be used in combination with an HCIC or HIC step (either before or after HCIC or HIC) include the use of anion exchange and/or cation exchange chromatography. Generally known commercially available anion exchange supports used in the purification of proteins bear quaternary ammonium functional groups. Preferred matrices for use in the present process are agarose or cellulose based matrices such as microcrystalline cellulose or cross-linked agaroses. Also particularly preferred are those matrices bearing diethyl aminoethyl, triethyl aminomethyl, or trimethyl aminomethyl functional groups. A particularly preferred anion exchange matrix is trimethyl aminomethyl crosslinked agarose, which is commercially available, e.g., Q-Sepharose Fast Flow® (Pharmacia). Generally known commercially available cation exchange supports that may be used in the purification of proteins bear acidic functionalities, including carboxy and sulfonic acids. Matrices containing the cation functionalities include various forms of celluloses and polystyrene based matrices. For example, weak cation exchangers known in the art include, but are not limited to, Carboxymethyl-Sepharose® and Carboxymethyl-Cellulose®. Strong cation exchangers known in the art include, but are not limited to,

sulfonated polystyrenes (AG 50W®, Bio-Rex 70®), sulfonated celluloses (SP-Sephadex®), and sulfonated Sepharoses (S-Sepharose®). A particularly preferred cation exchange matrix is S-Sepharose Fast Flow® (Pharmacia).

The chromatographic step involving these matrices is most preferably conducted as a column chromatography step or in alternative a batch absorptive technique, which optionally can be performed at a temperature between 25° C. to 40° C. Preferably, a salt is added to a washing or eluting buffer to increase the ionic strength of the buffer. Any of the salts conventionally used may be employed for this purpose as can be readily determined by one skilled in the art, with NaCl being one of the most frequently and conveniently used salts.

A conventional gel filtration step can also be used in combination with the HCIC or HIC chromatography process step. Representative examples of these matrices are polydextrans cross linked with acrylamides, such as composite hydrophilic gels prepared by covalently cross linking allyl dextran with N,N'-methylene bisacrylamide and crosslinked cellulose or agarose gels. Commercially available crosslinked dextran-acrylamides are known under the trade name Sephacryl® and are available from Pharmacia. Commercially available crosslinked dextran-agarose resins are known under the trade name Superdex®, available from Pharmacia. A preferred Superdex® gel is Superdex 200®. Examples of crosslinked cellulose gels are those commercially available cross linking porous cellulose gels, e.g., GLC 300® or GLC 1,000® that are available from Amicon Inc. Silica based resins such as TSK-Gel SW®, available from TosoHaas can be utilized. Polymer based resins such as TSK-Gel PW®, TSK Alpha Series®, Toyopearl HW packings® (copolymerization of ethylene glycol and methyl acrylate polymers) are also available from TosoHaas.

Preferably, HCIC or HIC can be combined with one or more of these ion exchange steps. When a combination of HCIC or HIC and various ion exchange or gel filtration steps are used, they can be performed in any order. For example, as described below a four step procedure can be followed which includes HCIC using hydrophobic charge induction chromatography material MEP HYPERCEL® or HIC using hydrophobic interaction chromatography material Macro-Prep Methyl®, then ion-exchange chromatography resins Q SEPHAROSE FAST FLOW®, SP SEPHAROSE FAST FLOW®, and lastly size-exclusion chromatography resin SUPERDEX 200®. Several of these procedures are set forth in more detail below.

MEP Hypercel Chromatography

MEP (mercaptoethylpyridine) Hypercel® (BioSeptra, Life Technologies) can be used for HCIC. It is a resin consisting of NEP linked to a regenerated cellulose bead of high porosity (80-100 microns). The functional group (MEP), consisting of a hydrophobic tail and an ionizable head group, is uncharged at neutral pH and can bind certain protein ligands based on hydrophobic interaction at a physiological ionic strength. Elution is accomplished by decreasing pH to 4 to 5, at which MEP is positively charged, and the protein elutes from the column due to electrostatic repulsion. For example, prepared harvest or harvest concentrate can be applied directly to the MEP column equilibrated with 25 mM sodium phosphate, pH 6.8, containing 180 mM sodium chloride and 2 mM DTT. Optionally, the column can then be washed with equilibration buffer containing 25 mM sodium caprylate until the absorbance at 280 nm (A280) stabilizes. The hmuGCB can be eluted from the column with 50 mM sodium acetate, 2 mM DTT, pH 4.7, and the peak as monitored at 280 nm can be collected.

MacroPrep Methyl Chromatography

An alternative to MEP Hypercel® is MacroPrep Methyl®, which is a hydrophobic interaction chromatography (HIC) resin. This resin consists of a methyl functional group attached to a bead composition of macroporous copolymerized glycol methacrylate and diethylene glycol dimethacrylate. For example, MacroPrep Methyl® (BioRad) chromatography can be performed as follows. The pH of the harvest or harvest concentrate is adjusted to 5.6, and ammonium sulfate is added to 0.70 M final concentration. The prepared harvest can be applied to the MacroPrep Methyl® column, which has been equilibrated in 0.70 M ammonium sulfate, 10 mM MES, pH 5.6. After application of the load, the column is washed with equilibrated buffer until the A280 returns to baseline. The hmGCB can be eluted with 10 mM MES, pH 5.6. The eluted hmGCB can be ultrafiltered and/or diafiltered in preparation for steps such as an ion exchange step such as Q Sepharose chromatography, SP Sepharose chromatography and/or Superdex 200 Chromatography.

Q Sepharose Chromatography

Q Sepharose Fast Flow® (Amersham Pharmacia) is a relatively strong anion exchange chromatography resin. The functional substituent is a quaternary amine group, which is positively charged over the working pH range of 2 to 12. Proteins with a net negative charge at the working pH will tend to bind to the resin at a relatively low ionic strength and can be eluted at higher ionic strength or lower pH. HmGCB does not bind to Q Sepharose at approximately pH 6 and low ionic strength, but impurities do bind, thereby purifying the sample. For example, the following protocol can be used to purify hmGCB in the sample by Q Sepharose Fast Flow® chromatography. Under appropriate conditions, hmGCB flows through this column, so the product is found in the flowthrough/wash fraction. Sodium phosphate (250 mM, pH 6) is added to the MEP elution pool prepared as described above to a final concentration of 25 mM, and the pH of the pool is adjusted to pH 6 with NaOH (and HCl if necessary). The conductivity is adjusted to 2.5±0.1 mS/cm by dilution with water or by ultrafiltration/diafiltration using 25 mM sodium phosphate, 2 mM DTT, at approximately pH 6. The material is then filtered and applied to a column of Q Sepharose Fast Flow® which has been equilibrated in 25 mM sodium phosphate, 2 mM DTT, pH 6.0. After application of the load, the column is washed with equilibration buffer until the A280 reaches baseline. The flowthrough/wash fraction can then be processed through another column, e.g., SP Sepharose Fast Flow® column, shortly thereafter, e.g., within 24 hours, or frozen and stored at about -20° C. to -80° C. prior to further processing.

Other strong anion exchange resins, such as MacroPrep High Q Support® (BioRad) can be used in place of Q Sepharose. A weaker anion exchange resin such as DEAE Sepharose Fast Flow® (Pharmacia) or MacroPrep DEAE® (BioRad) can also be used. The column is equilibrated in buffer, e.g., 25 mM sodium phosphate, pH 6. The pH of the sample is adjusted to pH 6 and the conductivity is adjusted by dilution or diafiltration to a relatively low ionic strength, which allows impurities to bind to the column and hmGCB to flow through. The sample is applied and the column is washed with equilibration buffer. Impurities are still bound to the column, and can be eluted with application of salt, e.g., sodium chloride or potassium chloride, or application of a lower pH buffer, or a combination of increased salt and lower pH.

The hmGCB can also be allowed to bind the anion exchange column during loading by decreasing the salt

concentration in the load or by running the column at a higher pH, or by a combination of both decreased salt and higher pH.

SP Sepharose Chromatography

SP Sepharose Fast Flow® (Amersham Pharmacia) is a relatively strong cation exchange chromatography resin. The functional substituent is a charged sulfonic acid group, which is negatively charged over a working pH range of 2 to 12. Proteins with a net positive charge at the working pH will tend to bind to the resin at a relatively low ionic strength and can be eluted at higher ionic strength or higher pH. HmGCB binds to SP Sepharose at approximately pH 6 and intermediate ionic strength (e.g., 6.5 mS/cm) and can be eluted at higher ionic strength (e.g., 10.7 mS/cm). Impurity proteins remain bound to SP Sepharose under conditions of hmGCB elution, thereby purifying the hmGCB in the sample. For example, the following protocol can be used to purify hmGCB by SP Sepharose Fast Flow® chromatography. Sodium chloride (2.0 M stock) is added to the Q Sepharose® flowthrough/wash until the conductivity is 6.3 mS/cm. The pH is checked and readjusted to pH 6.0 if necessary. Then, addition of sodium chloride stock is continued until the conductivity is 6.5 mS/cm. The material is filtered and applied to a column of SP Sepharose Fast Flow®, which has been equilibrated with 25 mM sodium phosphate, 44 mM sodium chloride, pH 6.0. After application of the load, the column is washed with equilibration buffer until the baseline is reached and eluted with 25 mM sodium phosphate, 84 mM sodium chloride, pH 6.0. HmGCB is found in the elution fraction.

Another cation exchange resin, e.g., Source 30S® (Pharmacia), CM Sepharose Fast Flow® (Pharmacia), MacroPrep CM Support® (BioRad) or MacroPrep High S Support® (BioRad), can be used as an alternative to SP Sepharose. The hmGCB can bind to the column at approximately pH 6 and low to intermediate ionic strength, such as 4 to 7 mS/cm. A buffer, e.g., 10 mM sodium citrate, pH 6.0, 10 mM MES, pH 6.0, 25 mM sodium phosphate, pH 6.0, or other buffer with adequate buffering capacity at pH 6.0 can be used to equilibrate the column. The ionic strength of the sample is adjusted by dilution or diafiltration to a level which will accommodate binding to the column. The sample is applied to the column and the column is washed after the load to remove unbound material. A salt, e.g., sodium chloride or potassium chloride, can be used to elute the hmGCB from the column. Alternatively, the hmGCB can be eluted from the column with a buffer of higher pH or a combination of higher salt concentration and higher pH.

The hmGCB can also be made to flow through the cation exchange column during loading by increasing the salt concentration in the equilibration buffer and in the sample load, by running the column at a higher pH or by a combination of both increased salt and higher pH.

Superdex 200 Chromatography

Superdex 200 prep grade® (Amersham Pharmacia) is used for size exclusion chromatography of hmGCB, whereby molecules are separated by size, molecular mass, Stokes radius or hydrodynamic volume. Superdex 200 is composed of dextran covalently cross linked to agarose and has a fractionation range of 10,000 to 60,000 molecular weight for globular proteins. For example, the following protocol can be used to purify hmGCB by Superdex 200® chromatography. The SP elution pool is concentrated by ultrafiltration using a 10,000 mw cutoff membrane. The concentrated pool is filtered, then applied to a Superdex 200 prep grade® column which has been equilibrated in 50 mM sodium citrate, pH 6.0. The A280 of the column effluent in

the initial fractions is collected and, for example, an 8 to 16% SDS polyacrylamide gel is run to determine pooling of fractions. Pooling may be decided based on visual inspection of the silver-stained gel.

Other size exclusion chromatography resins such as Sephacryl S-200 HR®, Bio-Gel A 1.5 m®, or Tosoh Haas TSK Gel resins can also be used to purify hmGCB. The buffer used for size exclusion chromatography of hmGCB is 50 mM sodium citrate, pH 6.0. Other buffers can also be used such as 25 mM sodium phosphate, pH 6.0 containing 0.15 M sodium chloride. The pH of the buffer can be between pH 5 and pH 7 and should have sufficient ionic strength to minimize ionic interactions with the column.

Variations of pH, buffer and/or salt concentration in any of the purification protocols described above can be performed by routine methods to achieve the desired purified product.

Assays for Determining Macrophage Uptake and Cellular Targeting of hmGCB

The uptake efficiency of hmGCB by macrophages can be determined by assaying, e.g., protein levels and/or enzyme activity in macrophages. For example, as described in the Examples below and in Diment et al. (1987) *J. Leukocyte Biol.* 42:485-490, an in vitro assay using a macrophage cell line can be used to determine absolute and mannose receptor specific uptake of hmGCB.

In addition, in vivo comparison of uptake of hmGCB and GCB by liver cells can be determined as described, for example, in Friedman et al. (1999) *Blood* 93:2807-2816. Briefly a mouse model can be injected with hmGCB or GCB, and then sacrificed shortly thereafter. The liver of the animal can then be used to prepare a suspension of liver cells, e.g., parenchymal cells, Kupffer cells, endothelial cells and hepatocytes. The cells can then be separated, identified by morphology and the protein levels and/or enzymatic activity of hmGCB and GCB in the various liver cell types can be determined. Alternatively, immunohistochemical detection may be used to localize hmGCB to a specific cell or cell type in tissue of treated animals.

Pharmaceutical Compositions

High mannose glucocerebrosidase (hmGCB) can be incorporated into pharmaceutical compositions suitable for administration to a subject, e.g., a human. The composition can include a sufficient dosage of hmGCB to treat a subject having Gaucher disease. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, excipients, dispersion media, coatings, antibacterial and antifungal agents, isotonic and adsorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, such media can be used in the compositions of the invention. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, and subcutaneous administration. Preferably, the route of administration is intravenous. Solutions or suspensions used for parenteral application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or

sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders, e.g., lyophilized preparations, for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL® (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged stability of the injectable compositions can be brought about by including in the composition an agent which delays adsorption, for example, aluminum monostearate, human serum albumin and gelatin.

Sterile injectable solutions can be prepared by incorporating the hmGCB in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying, e.g., lyophilization, which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811.

Treatment of Gaucher Disease

HmGCB, e.g., any hmGCB molecule or preparation described herein, can be used to treat a subject having Gaucher disease. Alternatively, any mannosidase knockout cell described herein, can be introduced into a subject having Gaucher disease to deliver hmGCB to the subject. Various routes of administration and various sites can be used. Once implanted in individual, the knockout cell can produce hmGCB.

Preferably, the knockout cells used will generally be patient-specific genetically engineered cells. It is possible, however, to obtain cells from another individual of the same species or from a different species. Use of such cells might require administration of an immunosuppressant, alteration of histocompatibility antigens, or use of a barrier device to prevent rejection of the implanted cells.

Gaucher disease is an autosomal recessive lysosomal storage disorder characterized by a deficiency in the lysosomal enzyme, glucocerebrosidase (GCB). GCB hydrolyzes the glycolipid glucocerebroside that is formed after degradation of glycosphingolipids in the membranes of white blood cells and red blood cells. If GCB hydrolysis is insufficient then glucocerebroside can accumulate in macrophages (Gaucher cells), causing anemia, thrombocytopenia, organomegaly and major bone problems.

There are several types of Gaucher disease including Gaucher type I, type II and type III, which can arise due to various mutations in the GCB gene. A "therapeutically effective amount" of hmGCB, i.e., a dosage of hmGCB sufficient to treat Gaucher disease, can be given to a subject having this disorder. The term "treat" as used herein refers to reducing or inhibiting one or more symptoms of Gaucher disease. Symptoms of Gaucher disease type I include: skeletal complications such as bone pain, bone lesions, osteopenia, osteonecrosis, avascular necrosis and pathological fractures; anemia; hepatosplenomegaly; splenic nodules and liver dysfunction; thrombocytopenia; and/or delayed growth and pubertal development. Symptoms of Gaucher disease type II include the symptoms of Gaucher type I as well as neck rigidity, apathy, catatonias, strabismus, increased deep reflex and laryngeal spasm. Symptoms of Gaucher disease type III are similar to Gaucher type II except milder and later in onset.

A therapeutically effective amount of hmGCB can be determined on an individual basis and will be based, at least in part, on consideration of the size of the patient, the agent used, the type of delivery system used, the time of administration relative to the severity of the disease, and whether a single, multiple, or a controlled release dose regimen is employed. Preferably, the dosage of hmGCB sufficient to treat Gaucher disease is less than the dosage of human tissue derived or human placenta derived GCB, or GCB produced by cells in vitro and then trimmed to expose core mannose residues.

Treatment of Other Lysosomal Storage Diseases

Generally, the invention described herein can be used to produce proteins for targeting any cells that express mannose receptors on their surface. Thus, the invention described herein can be used to treat any disorder in which it is desirable to target a protein for treatment to a mannose receptor-expressing cell. For example, the invention described herein can also be applied to other lysosomal storage enzymes and other lysosomal storage diseases in which cells, e.g., the cells of reticuloendothelial origin, accumulate undigested substrate. Reticuloendothelial cells include macrophages, Kupfer cells in the liver and histio-

cytes in the spleen. Such lysosomal storage diseases include, but are not limited to, Farber disease and Neimann-Pick disease.

Farber disease is an autosomal recessive lysosomal storage disorder characterized by a deficiency in acid ceramidase. Ceramidases are enzymes responsible for degradation of ceramide. If ceramide degradation is insufficient then ceramide accumulates leading to granuloma formation and histiocytic response. (Moser, H. W. Ceramidase deficiency: Farber lipogranulomatosis; In: *The Metabolic and Molecular Basis of Inherited Disease* (C. R. Scriver, A. L. Beaudet, W. S. Sly and D. Valle, Eds.), Seventh edition, pp. 2589-2599, McGraw-Hill Inc., New York (1995))

There are several types of Farber disease including Farber type I, type II, type III, type IV, and type V which differ in severity and sites of major tissue involvement. There is also type VI and type VII Farber disease. High mannose acid ceramidase can be given to a subject having Farber disease to treat, i.e., alleviate or reduce at least one symptom, of the disease. Symptoms of Farber disease type I include: swelling of the joints (particularly the interphalangeal, metacarpal, ankle, wrist, knee and elbow), palpable nodules in relation to the affected joints and over pressure points, a hoarse cry that may progress to aphonia, feeding and respiratory difficulty, poor weight gain and intermittent fever. The symptoms usually occur between ages two weeks and four months. Symptoms of Farber type II and type III include: subcutaneous nodules, joint deformities, and laryngeal involvement. These subjects survive longer than subjects having Farber type I. Farber disease type V symptoms include psychomotor deterioration beginning at one to two and half years of age.

Neimann-Pick disease type A and type B are an autosomal recessive lysosomal storage disorder characterized by a deficiency acid sphingomyelinase. Acid sphingomyelinase is an enzyme responsible for degradation of sphingomyelin. If sphingomyelinase is deficient, sphingomyelin and other lipids can accumulate in the monocyte-macrophage system. (Schuman, E. H. and Desnick, R. J. Neimann-Pick Disease types A and B: acid sphingomyelinase deficiencies; In: *The Metabolic and Molecular Basis of Inherited Disease* (C. R. Scriver, A. L. Beaudet, W. S. Sly and D. Valle, Eds.), Seventh edition, pp. 2589-2599, McGraw-Hill Inc., New York (1995))

There are several types of Neimann-Pick disease including type A and type B. High mannose acid sphingomyelinase can be given to a subject having Neimann-Pick disease to treat, i.e., alleviate or reduce at least one symptom, of the disease. Symptoms of Neimann-Pick disease type A include: enlargement of the spleen and liver, lymphadenopathy, microcytic anemia, decreased platelet count, hypotonia, muscular weakness, psychomotor retardation. Symptoms of Neimann-Pick type B include: enlargement of the liver and/or spleen, hepatosplenomegaly; pulmonary compromise.

Thus, high mannose lysosomal storage enzymes such as high mannose acid ceramidase or high mannose acid sphingomyelinase can be produced by the methods described herein in order to target these proteins to mannose receptor-expressing cells.

EXAMPLES

In experiments with HT-1080 cells in which the glucocerebrosidase gene has been activated as described in U.S. 5,641,670 (Gene-Activated™ GCB (GA-GCB)), the cells were treated with either kifunensine or swainsonine at concentrations ranging from 0.1 to 2 µg/mL.

Effect of Kifunensine or Swainsonine on GA-GCB Glycoforms

HT-1080 cells producing GA-GCB were plated in duplicate 6-well plates and the Production Medium adjusted to the following concentrations of kifunensine or swainsonine: 0 (no drug), 0.1, 0.25, 0.5, 1, and 2 $\mu\text{g/mL}$. The medium was harvested and the cells refed every 24 hours for three days. The samples from the third day were subjected to isoelectric focusing (IEF) analysis. The effect of kifunensine and swainsonine on the molecular charge of GA-GCB is shown by the IEF analysis. With both drugs, a concentration dependent increase in the apparent isoelectric point (pI) was observed, with kifunensine causing a much larger shift in pI than swainsonine at the highest concentration tested (2 $\mu\text{g/mL}$).

Effect of Kifunensine or Swainsonine on GA-GCB Production

Ten roller bottles (surface area, 1700 cm^2 each) were seeded in Growth Medium (DMEM with 10% calf serum) with HT-1080 cells producing GA-GCB. Following two weeks of growth, the medium was aspirated and 200 mL of fresh Production Medium (DMEM/F12, 0% calf serum) was added to three sets of roller bottles. Two sets of 4 roller bottles were treated with 1 $\mu\text{g/mL}$ of either kifunensine or swainsonine. The third group of two roller bottles received no drug treatment. After approximately 24 hours, the medium from each roller bottle was harvested, pooled and a sample taken for GA-GCB enzymatic activity analysis. This procedure was repeated for seven days. Stable production of GA-GCB was observed for all roller bottles throughout the seven daily harvests (Table 1). Absolute levels of the enzyme, however, varied according to drug treatment group with the following average GA-GCB production levels observed across the seven harvests: 38.3 ± 3.5 mg/L (control, no drug treatment), 24.5 ± 4.0 mg/L (swainsonine, 1 $\mu\text{g/mL}$), and 21.3 ± 2.8 mg/L (kifunensine, 1 $\mu\text{g/mL}$). Both drugs, therefore, resulted in stable, but lower production levels with the largest decrease seen for kifunensine (44% reduction relative to control).

TABLE 1

Roller Bottle Production of Glucocerebrosidase in Cells Treated with Mannosidase Inhibitors								
Treatment	Glucocerebrosidase ^{a)} Activity (^{b)} mg/Liter)							Average \pm Standard Deviation
	Harvest 1	Harvest 2	Harvest 3	Harvest 4	Harvest 5	Harvest 6	Harvest 7	
No drug added	35.8	36.6	44.9	40.5	34.6	38.3	37.2	38.3 ± 3.5
Swainsonine (1 $\mu\text{g/mL}$)	28.6	17.4	28.5	27.0	22.9	25.0	22.3	24.5 ± 4.0
Kifunensine (1 $\mu\text{g/mL}$)	26.0	22.9	17.7	21.2	18.4	21.0	22.0	21.3 ± 2.8

^{a)} Assay performed as follows: test article is mixed with the enzyme substrate (4-methylumbelliferyl- β -D-glucopyranoside) and incubated for 1 hour at 37° C. The reaction is stopped by the addition of NaOH/Glycine buffer. Fluorescence is quantified by the use of a fluorescence spectrophotometer.

^{b)} Specific activity: 2,500 Units/mg. One unit is defined as the conversion of 1 μMole of substrate in 1 hour at 37° C.

Effect of Kifunensine or Swainsonine on GA-GCB Uptake into Macrophages

GA-GCB produced in HT-1080 cells was used in an in vitro assay to determine uptake efficiency in a mouse macrophage cell line. The specific objective of the experiment

was to determine the absolute and mannose receptor-specific uptake of GA-GCB in mouse J774E cells. One day prior to assay, J774E cells were plated at 50,000 cells/ cm^2 in 12 well plates in Growth Medium. For the assay, 0.5 mL of Production Medium (DMEM/F12, 0% calf serum) containing 50 nM vitamin D3 (1,25-Dihydroxy vitamin D3) was added to the cells. Unpurified GA-GCB (from harvest 4, Table 1) was added to the test wells at a final concentration of 10 $\mu\text{g/mL}$ in the presence or absence of 2 $\mu\text{g/mL}$ mannan (a competitor for the mannose receptor). Three different forms of GA-GCB were used: GA-GCB from cells treated with kifunensine (1 $\mu\text{g/mL}$), GA-GCB from cells treated with swainsonine (1 $\mu\text{g/mL}$), and GA-GCB (1 $\mu\text{g/mL}$) from untreated cells. Control wells received no GA-GCB. The wells were incubated for 4 hours at 37° C., then washed extensively in buffered saline, scraped into GA-GCB enzyme reaction buffer, passed through 2 freeze/thaw cycles, and clarified by centrifugation. The supernatant was then quantitatively tested for enzyme activity and total protein. Internalization of GA-GCB into mouse J774E cells is shown in Table 2 and is reported as Units/mg of cell lysate. These results demonstrated that uptake of GA-GCB from kifunensine treated cells was 14-fold over background and 73% inhibitable by mannan and that uptake of GA-GCB from swainsonine treated cells was 7-fold over background and 67% inhibitable by mannan. In addition, they also demonstrate that uptake of GA-GCB from untreated cells was approximately 3-fold over background and 53% inhibitable by mannan. Thus, the inhibition of intracellular mannosidases by either kifunensine or swainsonine results in GA-GCB that can be transported into cells efficiently via the mannose receptor, with kifunensine causing an approximately 2-fold greater uptake than swainsonine. Improvement in targeting of GA-GCB to cells via mannose receptors can therefore be optimized by production of GA-GCB in the presence of kifunensine or swainsonine.

TABLE 2

Internalization of Glucocerebrosidase Into J774E Cells.
Glucocerebrosidase Produced from Cells Treated with Mannosidase Inhibitors

^{a)} Sample	^{a)} Glucocerebrosidase Activity (Units/mg cell lysate)		
	Absolute	Background Corrected	Inhibition (%)
Background (no GA-GCB added)	655	0	—
GA-GCB from untreated cells +	2816	2161	—
Mannan	1678	1023	53
GA-GCB from kifunensine treated cells + Mannan	9185	8530	—
GA-GCB from swainsonine treated cells + Mannan	2977	2322	73
GA-GCB from swainsonine treated cells + Mannan	4787	4132	—
	2036	1381	67

^{a)} Assay performed as follows: sample is mixed with the enzyme substrate (4-methylumbelliferyl- β -D-glucopyranoside) and incubated for 1 hour at 37° C. The reaction is stopped by the addition of NaOH/Glycine buffer. Fluorescence is quantified by the use of a fluorescence spectrophotometer. Total protein determined in freeze/thaw cell lysates by bicinchoninic acid (BCA). Activity reported as units/mg total protein. One Unit is defined as the conversion of 1 μMole of substrate in 1 hour at 37° C.

^{b)} Cells treated with drug received 1 $\mu\text{g/mL}$ of either Kifunensine or Swainsonine in the presence or absence of mannan (2 $\mu\text{g/mL}$).

Purification and Characterization of hmGCB

HmGCB was purified from the culture medium of human fibroblasts grown in the presence of kifunensine at a con-

centration of 2 µg/ml. The four N-linked glycans present on hmGCB were released by peptide N-glycosidase F and purified using a Sep-pak C18 cartridge. Oligosaccharides eluting in the 5% acetic acid fraction were permethylated using sodium hydroxide and methyl iodide, dissolved in methanol:water (80:20), and portions of the permethylated glycan mixture were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF-MS). The sample was analyzed on a Voyager STR Biospectrometry Research Station laser-desorption mass spectrometer coupled with Delayed Extraction using a matrix of 2,5-dihydroxybenzoic acid. A pattern of pseudomolecular ions is seen in the range m/z 1500–2500, indicating the presence of high-mannose glycans ranging from Man₅GlcNAc₂ to Man₉GlcNAc₂.

TABLE 3

Summary of Data Obtained from MALDI-TOF-MS Analysis of N-Glycans from hmGCB from Kifunensine-Treated Cells

M/Z	Peak Assignment	Approximate % of Total Glycans
1580	Man ₅ GlcNAc ₂	1.3
1730	Man ₆ GlcNAc ₂	11.2
1752		
1784		
1934	Man ₇ GlcNAc ₂	23.3
1957		
1988		
2139	Man ₈ GlcNAc ₂	32.0
2161		
2192		

TABLE 3-continued

Summary of Data Obtained from MALDI-TOF-MS Analysis of N-Glycans from hmGCB from Kifunensine-Treated Cells

M/Z	Peak Assignment	Approximate % of Total Glycans
2343	Man ₉ GlcNAc ₂	31.2
2365		
2397		
2969	Biantennary complex	1.0

The most abundant high mannose glycans present are Man₉GlcNAc₂ and Man₈GlcNAc₂, with decreasing abundances of Man₇GlcNAc₂, Man₆GlcNAc₂, and Man₅GlcNAc₂. A trace amount of a fucosylated biantennary complex glycan containing two sialic acid residues was observed. An approximate indication of the relative abundance of each glycan is obtained by measuring the peak heights. See Table 3. A more accurate assessment of the average chain length of the high mannose glycans was obtained by MALDI-TOF-MS analysis of the intact glycoprotein. A sharp peak was obtained at m/z 62,483.1 due to the homogeneity of the glycan chains. The mass of the mature peptide calculated from the amino acid sequence is 55,577.6, indicating the four N-linked glycan chains contribute 6905.5 to the total mass of hmGCB. From this number, it can be calculated that the average glycan length is 8.15 mannose residues.

All patents and references cited herein are incorporated in their entirety by reference.

Other embodiments are within following claims.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 2

<210> SEQ ID NO 1

<211> LENGTH: 8850

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 1

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What is claimed:

1. A method of producing a preparation of high mannose 60
 glucocerebrosidase (hmGCB) comprising a carbohydrate
 chain having at least four mannose residues, comprising:
 providing a mammalian cell that expresses a human
 glucocerebrosidase (GCB);
 contacting the cell with kifunensine;
 allowing the cell to produce hmGCB; and

harvesting the hmGCB from the cell or its culture media,
 to thereby produce an hmGCB preparation.

2. The method of claim 1, wherein removal of one or more
 α 1,2 mannose residue(s) distal to the pentasaccharide core
 is prevented.

3. The method of claim 1, wherein the kifunensine is
 65 present at a concentration between about 0.05 to 20.0 μg/ml.

4. The method of claim 3, wherein the kifunensine is
 present at a concentration between about 0.1 to 2.0 μg/ml.

5. The method of claim 1, further comprising contacting the cell with a class 2 processing mannosidase inhibitor.

6. The method of claim 5, wherein the class 2 processing mannosidase inhibitor is selected from the group consisting of: swainsonine, mannosatin, 6-deoxy DIM, 6-deoxy-6-fluoro-DIM and combinations thereof.

7. The method of claim 5, wherein the class 2 processing mannosidase inhibitor is swainsonine.

8. The method of claim 5, wherein the class 2 processing mannosidase inhibitor is present at a concentration between 0.05 to 20.0 $\mu\text{g/ml}$.

9. The method of claim 1, wherein the hmGCB has at least one carbohydrate chain having five mannose residues.

10. The method of claim 1, wherein the hmGCB has at least one carbohydrate chain having eight mannose residues.

11. The method of claim 1, wherein the hmGCB has at least one carbohydrate chain having nine mannose residues.

12. The method of claim 1, wherein the removal of one or more mannose residues distal to the pentasaccharide core is prevented on at least two carbohydrate chains of hmGCB.

13. The method of claim 1, wherein at least 60% of the hmGCB of the preparation have one or more carbohydrate chains in which the removal of one or more mannose residues distal to the pentasaccharide core has been prevented.

14. The method of claim 13, wherein the removal of three or more mannose residues distal to the pentasaccharide core has been prevented.

15. The method of claim 1, wherein at least about 20% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.

16. The method of claim 15, wherein at least about 40% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.

17. The method of claim 16, wherein at least about 60% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.

18. The method of claim 1, wherein at least about 80% or more of the carbohydrate chains of the hmGCB preparation have six or more mannose residues.

19. The method of claim 1, wherein the cell is a human cell and is a knockout for a class 2 processing mannosidase.

20. The method of claim 1, wherein the cell is a human cell and comprises a class 2 processing mannosidase antisense molecule.

21. The method of claim 1, wherein the cell comprises an exogenous nucleic acid sequence comprising a GCB coding region.

22. The method of claim 21, wherein the cell further comprises an exogenous regulatory sequence which functions to regulate expression of the GCB coding region.

23. The method of claim 1, wherein the cell comprises an exogenous regulatory sequence which functions to regulate expression of an endogenous GCB coding sequence.

24. The method of claim 1, wherein the cell is a primary cell.

25. The method of claim 1, wherein the cell is a secondary cell.

26. The method of claim 1, wherein the cell is a human cell.

27. The method of claim 26, wherein the cell is a fibroblast or a myoblast.

28. The method of claim 26, wherein the cell is an immortalized cell.

29. The method of claim 27, wherein the cell is an HT-1080 cell.

30. The method of claim 1, wherein the cell is contacted with kifunensine in culture media.

31. The method of claim 30, wherein the hmGCB is obtained from the media in which the cell is cultured.

32. A method of producing a preparation of high mannose glucocerebrosidase (hmGCB) comprising a carbohydrate chain having at least four mannose residues, the method comprising:

providing a human cell into which a nucleic acid sequence comprising an exogenous regulatory sequence has been introduced such that the regulatory sequence is operably linked to, and regulates the expression of, an endogenous GCB coding region;

contacting the cell with a class 1 mannosidase inhibitor such that the removal of at least one mannose residue distal to the pentasaccharide core of a precursor oligosaccharide of GCB is prevented; and

allowing the cell to produce hmGCB, to thereby produce an hmGCB preparation.

33. The method of claim 32, wherein the mannosidase inhibitor prevents the removal of one or more α 1,2 mannose residue(s) distal to the pentasaccharide core.

34. The method of claim 32, wherein the mannosidase inhibitor further prevents the removal of one α 1,3 mannose residue distal to the pentasaccharide core.

35. The method of claim 32, wherein the mannosidase inhibitor further prevents the removal of one α 1,6 mannose residue distal to the pentasaccharide core.

36. The method of claim 32, wherein the class 1 processing mannosidase inhibitor is kifunensine.

37. The method of claim 36, wherein the kifunensine is present at a concentration between about 0.05 to 20.0 $\mu\text{g/ml}$.

38. The method of claim 37, wherein the kifunensine is present at a concentration between about 0.1 to 2.0 $\mu\text{g/ml}$.

39. The method of claim 32, wherein the cell is further contacted with a class 2 mannosidase inhibitor.

40. The method of claim 39, wherein the class 2 processing mannosidase inhibitor is selected from the group consisting of: swainsonine, mannosatin, 6-deoxy DIM, 6-deoxy-6-fluoro-DIM and combinations thereof.

41. The method of claim 39, wherein the class 2 processing mannosidase inhibitor is swainsonine.

42. The method of claim 39, wherein the class 2 processing mannosidase inhibitor is present at a concentration between 0.05 to 20.0 $\mu\text{g/ml}$.

43. The method of claim 32, wherein the cell is a knockout for a class 2 processing mannosidase.

44. The method of claim 32, wherein the cell comprises a class 2 processing mannosidase antisense molecule.

45. The method of claim 32, wherein the hmGCB has at least one carbohydrate chain having six mannose residues of the precursor oligosaccharide.

46. The method of claim 32, wherein the hmGCB has at least one carbohydrate chain having eight mannose residues of the precursor oligosaccharide.

47. The method of claim 32, wherein the hmGCB has at least one carbohydrate chain having nine mannose residues of the precursor oligosaccharide.

48. The method of claim 32, wherein the mannosidase inhibitor prevents removal of at least three mannose residues distal to the pentasaccharide core of the precursor oligosaccharide of GCB.

49. The method of claim 32, wherein the mannosidase inhibitor prevents removal of one or more mannose residues distal to the pentasaccharide core on at least two of the carbohydrate chains of hmGCB.

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50. The method of claim 32, wherein at least 60% of the hmGCB of the preparation have one or more carbohydrate chains in which the removal of three or more mannose residues distal to the pentasaccharide core has been prevented.

51. The method of claim 32, wherein at least 20% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.

52. The method of claim 51, wherein at least 40% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.

53. The method of claim 52, wherein at least 60% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.

54. The method of claim 32, wherein at least about 80% or more of the carbohydrate chains of the hmGCB preparation have six or more mannose residues.

55. The method of claim 32, wherein the cell is a primary cell.

56. The method of claim 32, wherein the cell is a secondary cell.

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57. The method of claim 32, wherein the cell is a fibroblast or a myoblast.

58. The method of claim 32, wherein the cell is an immortalized cell.

59. The method of claim 58, wherein the cell is an HT-1080 cell.

60. The method of claim 36, wherein the cell is contacted with kifunensine in culture media.

61. The method of claim 60, wherein the hmGCB is obtained from the media in which the cell is cultured.

62. The method of claim 1, wherein the cell is a Chinese hamster ovary (CHO) cell transfected with an exogenous nucleic acid sequence comprising a human GCB coding sequence.

63. The method of claim 1, wherein the cell is a COS cell transfected with an exogenous nucleic acid sequence comprising a human GCB coding sequence.

* * * * *

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

**Assignee: Shire Human Genetic Therapies,
Inc.**

**Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS**

Attachment E

Maintenance Fee Statement



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PATENT NUMBER	FEE AMT	SUR CHARGE	PYMT DATE	U.S. APPLICATION NUMBER	PATENT ISSUE DATE	APPL. FILING DATE	PAYMENT YEAR	SMALL ENTITY?	ATTY DKT NUMBER
7,138,262	\$980.00	\$0.00	03/25/10	09/641,471	11/21/06	08/18/00	04	NO	HGT 0013

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

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Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment F

Brumshtein et al. (2010) Glycobiology 20(1):24-32

Characterization of gene-activated human acid- β -glucosidase: Crystal structure, glycan composition, and internalization into macrophages

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Received on June 1, 2009; revised on August 30, 2009; accepted on September 1, 2009

Gaucher disease, the most common lysosomal storage disease, can be treated with enzyme replacement therapy (ERT), in which defective acid- β -glucosidase (GlcCerase) is supplemented by a recombinant, active enzyme. The X-ray structures of recombinant GlcCerase produced in Chinese hamster ovary cells (imiglucerase, Cerezyme®) and in transgenic carrot cells (prGCD) have been previously solved. We now describe the structure and characteristics of a novel form of GlcCerase under investigation for the treatment of Gaucher disease, Gene-Activated™ human GlcCerase (velaglucerase alfa). In contrast to imiglucerase and prGCD, velaglucerase alfa contains the native human enzyme sequence. All three GlcCerase consist of three domains, with the active site located in domain III. The distances between the carboxylic oxygens of the catalytic residues, E340 and E235, are consistent with distances proposed for acid-base hydrolysis. Kinetic parameters (K_m and V_{max}) of velaglucerase alfa and imiglucerase, as well as their specific activities, are similar. However, analysis of glycosylation patterns shows that velaglucerase alfa displays distinctly different structures from imiglucerase and prGCD. The predominant glycan on velaglucerase alfa is a high-mannose type, with nine mannose units, while imiglucerase contains a chitobiose tri-mannosyl core glycan with fucosylation. These differences in glycosylation affect cellular internalization; the rate of velaglucerase alfa internalization into human macrophages is at least 2-fold greater than that of imiglucerase.

Keywords: Gaucher disease/gene activation/
glucocerebrosidase/glycans/mannose-6-phosphate receptor/
site-specific glycosylation/X-ray structure

Introduction

Gaucher disease is caused by mutations in the gene encoding the lysosomal enzyme, acid- β -glucosidase (glucocerebrosidase, GlcCerase, E.C. 3.2.1.45) (Beutler and Grabowski 2001;

Futerman and Zimran 2006). The most common treatment for Gaucher disease is enzyme replacement therapy (ERT), in which defective GlcCerase is supplemented with an active enzyme. ERT using imiglucerase, a recombinant analog of human GlcCerase expressed in Chinese hamster ovary (CHO) cells has been available for ~15 years. After expression and purification, imiglucerase is modified by exo-glycosidase treatment (Friedman and Hayes 1996) to expose the core mannose residues that can be recognized by macrophages. Glycan remodeling greatly improves targeting to and internalization by macrophages, the main cell type affected in Gaucher disease (Futerman and Zimran 2006). An alternative means of producing GlcCerase (prGCD) in transgenic carrot root cells has been developed (Aviezer et al. 2009). The X-ray structures of imiglucerase and prGCD have been previously reported (Dvir et al. 2003; Shaaltiel et al. 2007).

In the current study, we have used gene activation in a well-characterized, continuous human cell line to produce gene-activated human acid- β -glucocerebrosidase (velaglucerase alfa). Gene activation refers to targeted recombination with a promoter that activates the endogenous GlcCerase gene in the selected human cell line. Velaglucerase alfa is secreted as a monomeric glycoprotein of approximately 63 kDa and is composed of 497 amino acids with a sequence identical to that of the natural human protein (Zimran et al. 2007). Glycosylation of velaglucerase alfa is altered by using kifunensine, a mannosidase I inhibitor, during cell culture, which results in the secretion of a protein containing predominantly high-mannose type glycans (Elbein et al. 1990).

Herein we describe the crystal structure of velaglucerase alfa, using a preparation that had been partially deglycosylated, and show that it is similar to that of imiglucerase (Dvir et al. 2003) and prGCD (Shaaltiel et al. 2007). Velaglucerase alfa differs from imiglucerase and prGCD as the latter two enzymes contain a mutation at residue 495 (an Arg to His substitution: R495H), and prGCD contains seven additional residues at the C terminus (DLLVDTM) and two additional residues at the N terminus (EF). Moreover, the kinetic parameters and specific activity of velaglucerase alfa are very similar to those of imiglucerase. We also compare the glycosylation patterns of velaglucerase alfa and imiglucerase by use of LC-MS and assess the impact of the different glycosylation patterns by analyzing internalization in human macrophages.

Results and discussion

X-ray structure

Diffraction-quality crystals of velaglucerase alfa were obtained after partial deglycosylation using *N*-glycosidase F, by a procedure similar to that previously described for imiglucerase (Dvir et al. 2003). Velaglucerase alfa crystallized in the same space

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Table I. Data collection and refinement statistics

	Velaglucerase alfa
Data collection	
Space group	C222 ₁
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	109.37, 285.55, 91.69
<i>abg</i> (°)	90.00, 90.00, 90.00
Resolution (Å)	19.9–2.7 (2.75–2.70) ^a
<i>R</i> _{sym} (%)	15.7 (51.0)
<i>I</i> / <i>σ</i> (<i>I</i>)	14.4 (4.6)
Completeness (%)	100 (100)
Redundancy	7.5 (7.6)
Refinement	
Resolution (Å)	19.9–2.7
Number of reflections	39,776
<i>R</i> _{work} / <i>R</i> _{free}	17.3/23.4
Rms deviations	
Bond lengths (Å)	0.012
Bond angles (°)	1.486
Number of refined atoms	
Protein	7871
Carbohydrates	70
Ions	90
Solvent	326
Ramachandran outliers (%)	0.4

^aThe highest resolution shell is shown in parentheses.

group, C222₁, as imiglucerase (Table I), and unit cell parameters were similar to the previously published GlcCerase structures (Dvir et al. 2003; Premkumar et al. 2005; Brumshtein et al. 2006). The asymmetric unit contained two copies of velaglucerase alfa, designated as molecules A and B. The root mean square deviation (RMSD) value between molecules A and

B (<0.3 Å) shows that they are virtually identical. A comparison of the structures of imiglucerase, prGCD, and velaglucerase alfa demonstrates that these structures are very similar, with an RMSD of 0.35–0.46 Å (Table II).

Velaglucerase alfa thus consists of three noncontiguous domains, with the catalytic site located in domain III (residues 76–381 and 416–430), which is a (β/α)₈ (TIM) barrel (Figure 1). A more detailed analysis of the active site reveals that it is virtually identical to that of imiglucerase (Figure 2), with the distances between the carboxylic oxygens of the catalytic residues, E340 and E235 (5.2 Å in molecule A and 5.1 Å in molecule B), similar to those obtained previously (Brumshtein et al. 2006) and in agreement with the distances proposed for acid–base hydrolysis (Davies and Henriksat 1995). Moreover, the three loops (loop 1, residues 345–350; loop 2, residues 393–399; and loop 3, residues 312–319) observed in previous structures (reviewed in Kacher et al. (2008)) are also seen in velaglucerase alfa. Similarly to prGCD (Shaaltiel et al. 2007), loops 2 and 3 show differences in their backbone angles and side chain orientations in the two molecules of the asymmetric unit, whereas loop 1, since it makes crystal contacts, exhibits less pronounced conformational changes (Figure 2). In the case of loop 3, a helical conformation is seen in molecule B, whereas a coiled conformation is seen in molecule A (Figure 3), as previously reported for imiglucerase (Brumshtein et al. 2006). Although the crystal was cryo-protected with 25% ethylene glycol, we did not detect any ethylene glycol molecules in the electron density map.

Imiglucerase and prGCD both contain an Arg to His mutation at residue 495, with H495 making an H-bond (2.6 Å) with the peptide carbonyl of F31. In contrast, velaglucerase alfa contains a sequence identical to that of the natural human enzyme,

Table II. RMS deviations of velaglucerase alfa compared to imiglucerase and prGCD. RMS deviations (Å) are shown for each of the two copies of the molecules in the asymmetric unit and were calculated using PyMol (www.pymol.org). The PDB codes for imiglucerase and pr-GlcCerase are 2I25 and 2V3F, respectively

	Imiglucerase-A	Imiglucerase-B	prGCD-A	prGCD-B
Velaglucerase alfa-A	0.39	0.35	0.36	0.40
Velaglucerase alfa-B	0.38	0.43	0.46	0.46

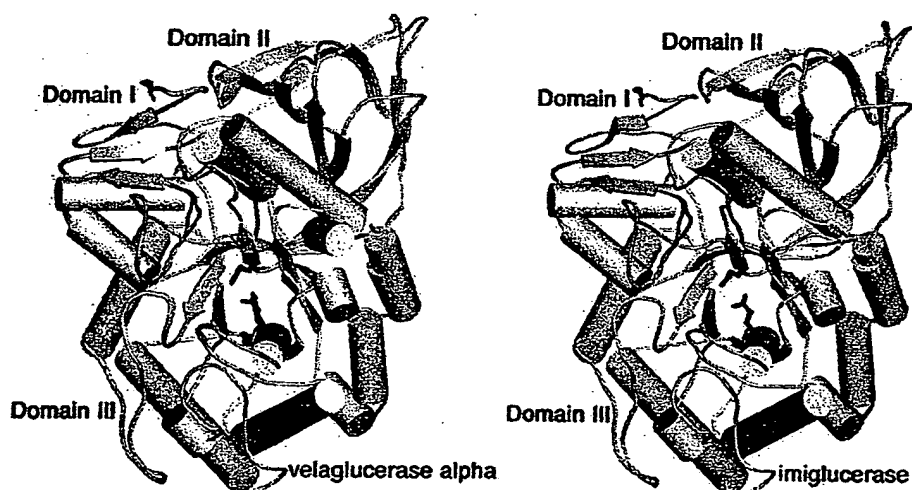


Fig. 1. Comparison of the crystal structures of velaglucerase alfa and imiglucerase. The three domains of the enzymes are colored pink (domain I, residues 1–29 and 383–414), blue (domain II, residues 30–75 and 431–497), and gray (domain III, residues 76–382 and 415–430).

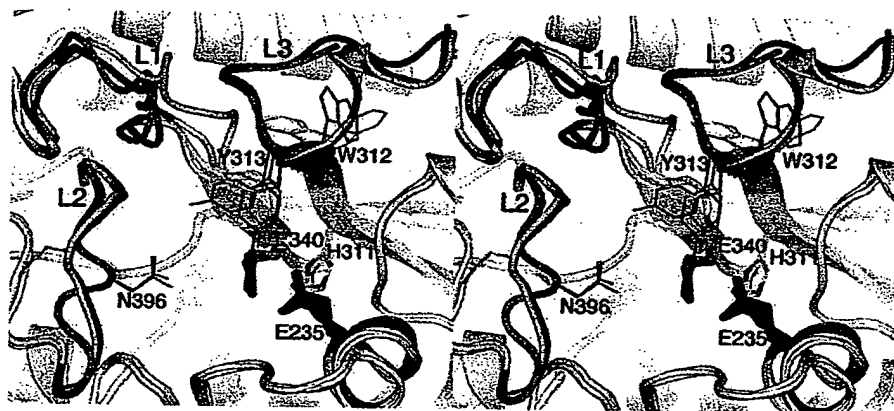


Fig. 2. Active site of velaglucerase alfa. Stereo representation of an overlay of the active sites of imiglucerase (blue and magenta) and velaglucerase alfa (yellow and green). Catalytic residues are shown as red sticks. Loops near the entrance to the active site are indicated (L1, loop 1; L2, loop 2; L3, loop 3).

with an Arg at residue 495, which does not make a similar H-bond. No major structural differences were observed in velaglucerase alfa around residue R495, relative to imiglucerase or prGCD. Two mutations which cause Gaucher disease, R496 and D474 (Figure 4) (Kawame et al. 1992; Beutler et al. 1993; Choy et al. 1998), are in close proximity to R495 near the N-terminus of GlcCerase. D474 is at the end of a β -strand, and R496 is part of a coil with no clear secondary structure, and their side-chains form a salt bridge and hydrogen bonds with each other; mutations in either of these two residues would disrupt these interactions. By analyzing the geometry and the interactions between the side chains of these two residues, and the secondary structure of the region, we conclude that R496 or D474 may be involved in stabilizing the conformation of the N-terminus of the enzyme by their side chain interactions, with disruption of these bonds resulting in a flexible N-terminus and hence in a less stable structure. However, neither of these residues interacts with R495.

Kinetic analysis

To further compare velaglucerase alfa and imiglucerase, and to determine if the mutation at residue 495 has any effect, kinetic parameters and specific activity were determined using a natural glucosylceramide (GlcCer) substrate, rather than a surrogate substrate typically used to assess enzyme activity. Velaglucerase alfa has a k_{cat} of 2100 min^{-1} , a K_m of $19 \text{ }\mu\text{M}$, and a V_{max} of $0.61 \text{ }\mu\text{M min}^{-1}$. Imiglucerase has a k_{cat} of 1900 min^{-1} , a K_m of $15 \text{ }\mu\text{M}$, and a V_{max} of $0.56 \text{ }\mu\text{M min}^{-1}$ (Figure 5). Similar K_m values were reported in the literature; GlcCerase derived from brain tissue and fibroblasts both have a K_m of $32 \text{ }\mu\text{M}$ using GlcCer from Gaucher spleen (Vaccaro et al. 1982), while imiglucerase and prGCD have a K_m of 15.2 and $20.7 \text{ }\mu\text{M}$, respectively, using a fluorescent GlcCer analog, C6-NBD-GlcCer (Shaaltiel et al. 2007). In addition, at a $210 \text{ }\mu\text{M}$ GlcCer substrate concentration, velaglucerase alfa and imiglucerase have similar specific activities of 26 and 24 U/mg , respectively. These results

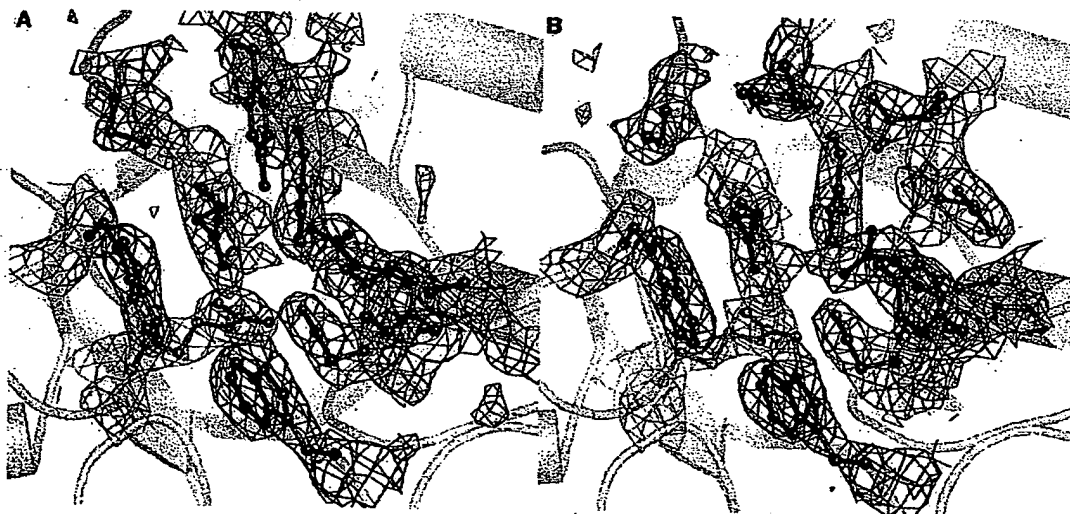


Fig. 3. Electron density around the catalytic center. Catalytic residues E235 and E340 are shown as red balls and sticks and surrounding residues are in dark gray. Contours of the $2F_o - F_c$ map are shown as a blue mesh (at 1.2σ); contours of the $F_o - F_c$ map are shown in green mesh (at 3σ) and in magenta (at -3σ). Several $F_o - F_c$ peaks are visible in the active site, but they did not overlap with the $2F_o - F_c$ map, nor are they continuous; hence, at this resolution they appear to be noise. A and B show the catalytic centers of molecules A and B, respectively, in the asymmetric unit.

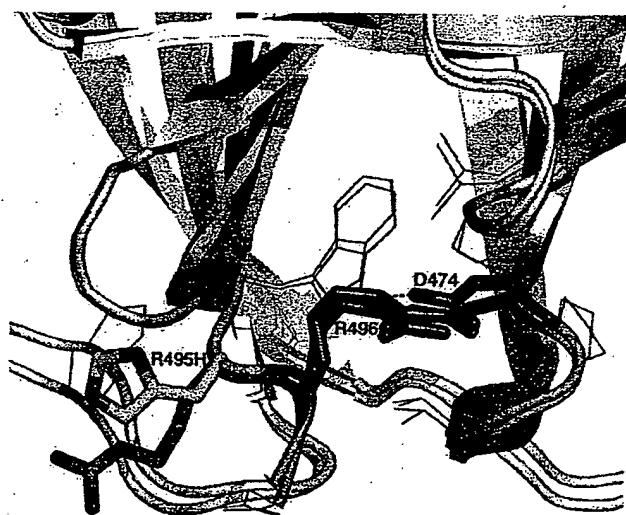


Fig. 4. Mutations at the C-terminus of GlcCerase. Imiglucerase and pr-GlcCerase contain a His at residue 495 (yellow), whereas velaglucerase alfa contains Arg (green). Mutations R496 and D474, which cause Gaucher disease, are shown in magenta. Residues within 4 Å distance of R495 and R496 are shown in cyan.

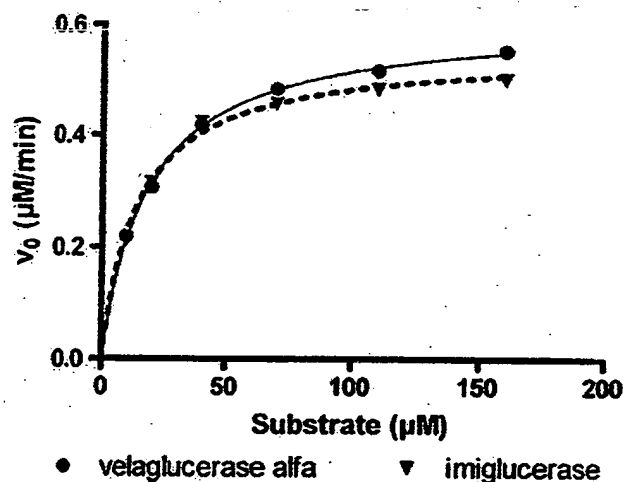


Fig. 5. Kinetic analysis of velaglucerase alfa and imiglucerase. V_{max} and K_m values were determined using a natural GlcCer substrate ($n = 2$).

demonstrate that human and CHO-cell derived GlcCerase, prepared by two different manufacturing processes, have similar enzymatic activities for the natural substrate.

Glycan composition

We next examined which sugars could be detected in the crystal structure of velaglucerase alfa. Even after partial deglycosylation using *N*-glycosidase F, two sugar residues were observed attached to residue N19 in both molecules A and B (Figure 6). One sugar was detected on N146 in molecule A whereas no sugars were detected on N146 in molecule B (Figure 6). As reported previously for imiglucerase, no sugars were detected attached to either N270 or N59 in velaglucerase alfa. It should

be noted that sugars attached to N270 have not been seen in any of the crystal structures solved to date, and sugars have been seen only occasionally on N59 (Brumshtein et al. 2006). The inability to detect sugars on either N59 or N270 is most likely due to the high flexibility of the corresponding glycan chains since nano-liquid chromatography electrospray ionization tandem mass spectrometry (nano-LC-ESI-MS/MS) analysis of intact imiglucerase (Kacher et al. 2008), and of velaglucerase alfa (see below) showed that glycan chains were attached to both these residues.

Velaglucerase alfa and imiglucerase bear distinctly different glycan chains due to the differences in their manufacture. In our comparative study of the carbohydrate content of unmodified velaglucerase alfa and imiglucerase by LC-ESI-MS, four of the five potential glycosylation sites, namely, N19, N59, N146, and N270, were observed to be fully occupied in both. As expected from the crystal structures, N462 is fully unoccupied in both, due to its buried location.

According to LC-ESI-MS analysis of glycopeptide maps, velaglucerase alfa contains primarily high-mannose type glycans, consisting of six to nine mannose units. Listed as the predominant structure in Table III, the most abundant ion present in the averaged spectra for each site corresponds to a glycan with nine mannose units. Glycan microheterogeneity was observed at each site and the less abundant structures are listed as other glycans. These other glycans consist of mannose residues with phosphorylation at the C-6 position to create a mannose-6-phosphate (M6P) residue. The lowest levels of M6P were at N19; N59 and N146 had similar but higher levels relative to N19, while N270 had the highest amount of M6P. Despite the site-specific variation in relative levels of M6P, nonphosphorylated glycans remained the predominant species for all four sites. Also observed on N59, N146 and N270 were mono-sialylated mono-antennary hybrid and complex-type structures with core fucosylation, which were quantified by glycan map analysis. These structures are consistent with a low percentage of glycosylation sites escaping kifunensine inhibition, resulting in glycan maturation and core fucosylation. In the case of hybrid-type glycans, only a single antenna matured.

The results from site-specific glycan characterization were corroborated by glycan map analysis (Figure 8), which demonstrates high-mannose type glycans consisting of six to nine mannose units with a predominant nine-mannose structure. Estimates from glycan map analysis show that the mono-sialylated mono-antennary hybrid structures account for ~2% of the total glycan pool. The map also demonstrates the presence of high-mannose glycans containing one GlcNAc-capped M6P, a result of incomplete glycan processing, as well as high-mannose glycans bearing a single M6P. Also consistent with these results were data obtained from monosaccharide compositional analysis that demonstrates approximately 0.8 mole of M6P per mole of velaglucerase alfa, and approximately 0.6 moles of M6P per mole of imiglucerase.

Site-specific glycan analysis demonstrated that imiglucerase contains primarily complex-type glycans with core fucosylation that terminate with the chitobiose tri-mannosyl core (Table IV), with an exception at the N19 site, which was observed to be devoid of fucose. These structures are as expected for GlcCerase with exoglycosidase treatment to expose the core mannose residues. Imiglucerase also contains glycan microheterogeneity at each site of glycosylation, with lower

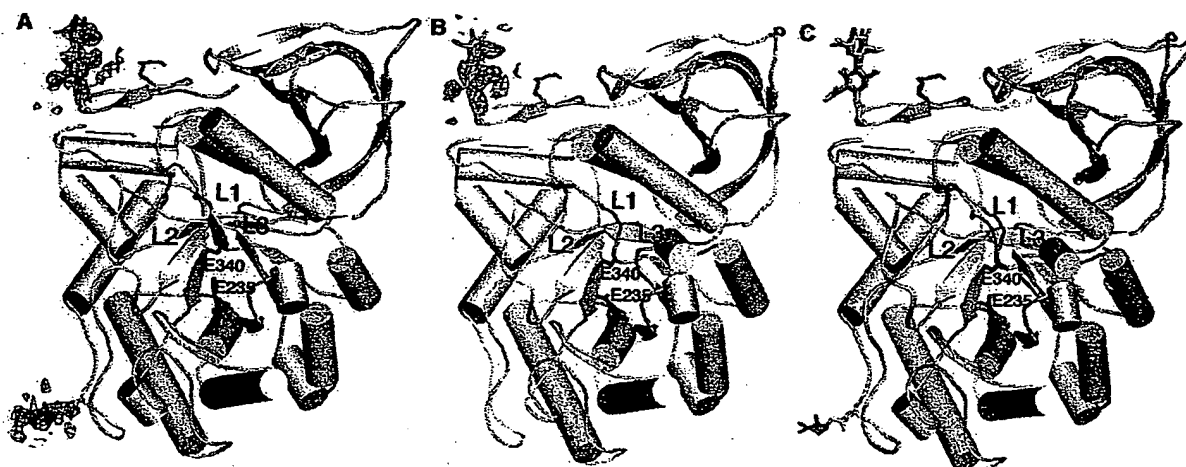


Fig. 6. Glycosylation sites seen in the crystal structure of velaglucerase alfa. $2F_o - F_c$ electron density maps are shown, which are contoured at 1.2 σ in the vicinity of two of the putative glycosylation sites, N19 and N146 for molecule A, and N19 for molecule B. (A) Glycosylation sites detected in molecule A are shown in green. (B) Glycosylation site detected in molecule B is shown in yellow. (C) Superposition of the two individual molecules in the asymmetric unit reveals their similarity. In all three representations, catalytic residues E235 and E340 are shown as red sticks.

Table III. Carbohydrate composition of velaglucerase alfa. Predominant structures are those observed to be most abundant at each *N*-linked glycosylation site. The other glycans consisted mostly of high-mannose type structures (some with M6P) and with the hybrid and complex types observed at low levels (~2% of the total as determined by glycan map analysis)

Glycosylation site	Predominant glycan	Other glycans
Asn19	High mannose (Man) ₉ (GlcNAc) ₂	High mannose (Man) ₆₋₈ (GlcNAc) ₂ Phosphorylated high mannose (Phos) ₁ (Man) ₈₋₉ (GlcNAc) ₂ GlcNAc-capped phosphate (Phos) ₁ (Man) ₈₋₉ (GlcNAc) ₃ Hybrid (Hex) ₂ (Man) ₃ (GlcNAc) ₃ (Fuc) ₁
Asn59	High mannose (Man) ₉ (GlcNAc) ₂	High mannose (Man) ₅₋₈ (GlcNAc) ₂ Phosphorylated high mannose (Phos) ₁ (Man) ₇₋₉ (GlcNAc) ₂ GlcNAc-capped phosphate (Phos) ₁ (Man) ₈₋₉ (GlcNAc) ₃ Hybrid (NeuAc) ₁ (Gal) ₁ (Man) ₅ (GlcNAc) ₃ (Fuc) ₁ Complex (NeuAc) ₀₋₂ (Gal) ₂ (Man) ₃ (GlcNAc) ₄ (Fuc) ₁ (Gal) ₃ (Man) ₃ (GlcNAc) ₅ (Fuc) ₁
Asn146	High mannose (Man) ₉ (GlcNAc) ₂	High mannose (Man) ₆₋₈ (GlcNAc) ₂ Phosphorylated high mannose (Phos) ₁ (Man) ₇₋₉ (GlcNAc) ₂ GlcNAc-capped phosphate (Phos) ₁ (Man) ₉ (GlcNAc) ₃ Hybrid (NeuAc) ₁ (Gal) ₁ (Man) ₅ (GlcNAc) ₃ (Fuc) ₁
Asn270	High mannose (Man) ₉ (GlcNAc) ₂	High mannose (Man) ₆₋₈ (GlcNAc) ₂ Phosphorylated high mannose (Phos) ₁ (Man) ₈₋₉ (GlcNAc) ₂ GlcNAc-capped phosphate (Phos) ₁ (Man) ₉ (GlcNAc) ₃ Hybrid (Gal) ₁ (Man) ₇ (GlcNAc) ₃ (Fuc) ₁ (NeuAc) ₁ (Gal) ₁ (Man) ₅ (GlcNAc) ₃ (Fuc) ₁ Complex (NeuAc) ₂ (Gal) ₂ (Man) ₃ (GlcNAc) ₄ (Fuc) ₁
Asn462	Not detected	Not detected

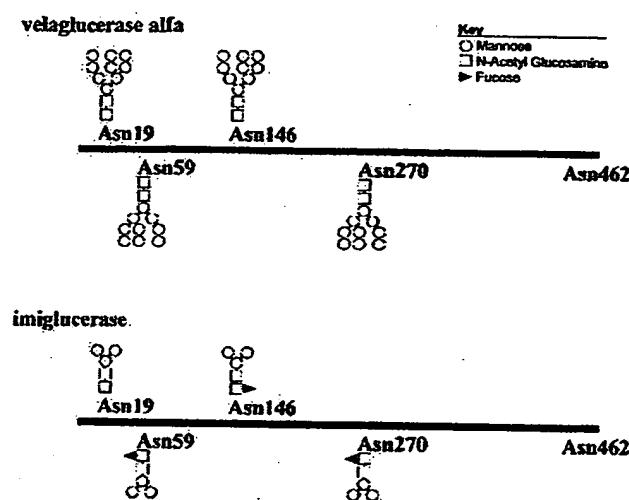


Fig. 7. Glycan structures of velaglycerase alfa and imiglycerase. Predominant *N*-linked carbohydrate structures on velaglycerase alfa (top) and imiglycerase (bottom) are shown graphically at their relative positions along the protein backbone.

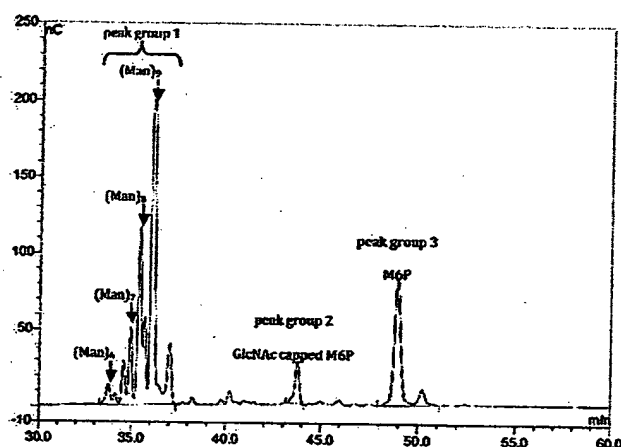


Fig. 8. Glycan map analysis of velaglycerase alfa. Glycans released by *N*-glycosidase F were analyzed by anion-exchange chromatography with amperometric detection. The method resolves glycans based on negative charge where peak group 1 corresponds to high-mannose type neutral glycans that are resolved into multiple peaks according to the number of mannose units, peak group 2 corresponds to high-mannose type glycans with one M6P that retained its GlcNAc cap (one negative charge), and peak group 3 corresponds to high-mannose type glycans containing one fully processed M6P (two negative charges). In peak group 1, smaller peaks are resolved that correspond to positional isomers of the various oligomannose types observed.

levels of core structures terminating with *N*-acetylglucosamine (GlcNAc) that are likely a result of incomplete digestion with *N*-acetylglucosaminidase. At N146 and N270, high-mannose type glycans were observed containing five to six mannose units with one M6P.

The glycan graphics shown in Figure 7 help to visualize the predominant structures for both forms of GlcCerase as described in Tables III and IV. These structures were consistent with glycan types and levels observed with glycan map analysis as well as with previous reports (Van Patten et al. 2007). In the current

Table IV. Carbohydrate composition of imiglycerase. Predominant structures are those observed to be most abundant at each *N*-linked glycosylation site. The other glycans consisted mostly of core structures with additional GlcNAc and high-mannose structures with M6P

Glycosylation site	Predominant glycan	Other glycans
Asn19	Complex (Man) ₃ (GlcNAc) ₂	Complex (Man) ₃ (GlcNAc) ₃
Asn59	Complex (Man) ₃ (GlcNAc) ₂ (Fuc) ₁	Complex (Man) ₃ (GlcNAc) ₃ (Fuc) ₁
Asn146	Complex (Man) ₃ (GlcNAc) ₂ (Fuc) ₁	Complex (Man) ₃ (GlcNAc) ₃₋₄ (Fuc) ₁ Phosphorylated high mannose (Phos) ₁ (Man) ₅₋₆ (GlcNAc) ₂ GlcNAc-capped phosphate (Phos) ₁ (Man) ₅₋₆ (GlcNAc) ₃
Asn270	Complex (Man) ₃ (GlcNAc) ₂ (Fuc) ₁	Complex (Man) ₃ (GlcNAc) ₃₋₄ (Fuc) ₁ Phosphorylated high -mannose (Phos) ₁ (Man) ₅₋₆ (GlcNAc) ₂ GlcNAc-capped phosphate (Phos) ₁ (Man) ₅₋₆ (GlcNAc) ₃

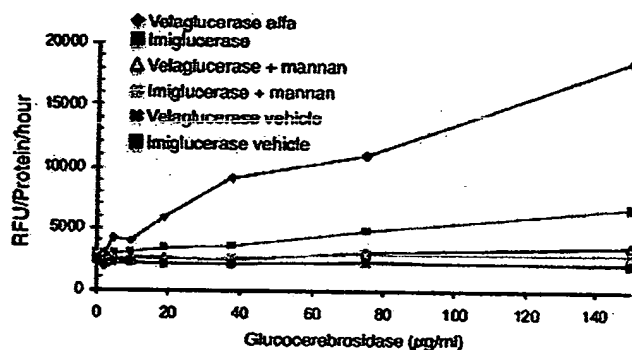


Fig. 9. Velaglycerase alfa and imiglycerase internalization into differentiated macrophages. The ordinate of the graph represents the fluorescence data normalized for the cellular protein concentration and incubation time (RFU/ μ g/h). The GlcCerase dose is shown on the abscissa.

study, the glycans of prGCD were not characterized, but earlier studies demonstrated the presence of core α -(1,2)-xylose and core α -(1,3)-fucose (Shaaltiel et al. 2007), which are unique to plant-derived proteins and would not be expected to be present on either velaglycerase alfa or imiglycerase.

Internalization by macrophages

Internalization of proteins by endocytosis is highly dependent upon their carbohydrate composition and has been well characterized (Kornfeld 1986). A comparison of the internalization rate of velaglycerase alfa to that of imiglycerase in U937-derived macrophages demonstrated that velaglycerase alfa is internalized approximately 2.5-fold more efficiently than imiglycerase (Figure 9). Internalization of both enzymes could be inhibited by the addition of mannan to the culture medium, demonstrating that internalization was mediated via mannose receptors; moreover, U937 cells were shown by immunohistochemistry to express mannose receptors (CD206) (data not shown). It should be noted that during optimization of this assay, variations in results were obtained when different culture media were used.

Therefore, additional research will be required to determine the exact nature of the uptake since different mannose receptors exist, which may be involved in this cellular internalization. In contrast, the addition of M6P to the culture medium had no effect, confirming that the M6P receptor is not involved in internalization (data not shown). Since velaglycerase α and imiglycerase display similar kinetic parameters, specific activities, and structural features, the different rates of internalization can be ascribed to differences in glycosylation patterns between velaglycerase α and imiglycerase, with the increased rate of internalization of velaglycerase α likely due to the expression of longer chain high-mannose type glycans compared to the core mannose structures found on imiglycerase.

Conclusions

In summary, the X-ray structure of velaglycerase α is very similar to those of recombinant GlcCerases produced in other expression systems, with the R495H mutations found in imiglycerase and pGCD having no effect on the secondary structure. The main difference between imiglycerase and velaglycerase α concerns their glycan structures, with the latter containing longer chain high-mannose type glycans compared to the core mannose structures found on imiglycerase. This difference in glycosylation appears to lead to the increased cellular uptake of velaglycerase α over imiglycerase. The role of protein glycosylation in cellular uptake is widely established in many cell types (Barton et al. 1991). However, while the function of the macrophage mannose receptor (MR; CD206) in internalization of mannoseylated proteins is well characterized (East and Isacke 2002), a growing family of carbohydrate-binding receptors have been implicated in diverse macrophage functions including removal and disposal of endotoxin (Ono et al. 2006), utilization of secreted lysosomal enzymes (Abe et al. 2008), phagocytosis (Kang et al. 2005), and regulation of the innate immune response to microbial pathogen-associated structures (Gamer et al. 1994). Thus, the differences in uptake observed between imiglycerase and velaglycerase- α can be attributed to differences in affinity for CD206, or alternatively could be due to differential uptake mediated by other macrophage mannose receptors such as Endo180. This observed increase in cellular uptake of velaglycerase- α over imiglycerase can be envisioned to lead to a more rapid time to improvement of clinical parameters and potentially increased therapeutic efficacy.

Material and methods

Crystallization, structure determination, and refinement

Velaglycerase α was partially deglycosylated (Kacher et al. 2008) prior to crystallization, as previously described for imiglycerase (Dvir et al. 2003; Premkumar et al. 2005), using *N*-glycosidase F (88 h at 25°C), which removes carbohydrate chains from proteins and peptides by cleaving the amide bonds between Asn residues and *N*-acetylglucosamine (GlcNAc) (Han and Martinage 1992), but does not necessarily remove all carbohydrate chains from native proteins. Subsequent to *N*-glycosidase F-treatment, velaglycerase α was diluted in the crystallization buffer (10 mM citrate pH 5.5, 7% (v/v) ethanol, 0.02% (w/v) Na₂S₂O₃) and passed through a Centricon YM-30 centrifugal filter device with a molecular mass cut-off of ~30 kDa, to give a final concentration of 4–5 mg/mL. Ve-

laglycerase α crystals were obtained by micro-batch crystallization under oil (Chayen et al. 1990) using a Douglas Instruments Oryx6 robot. The crystallization solution had a 1:1 ratio of the concentrated enzyme solution and of 1 M (NH₄)₂SO₄/0.1 M HEPES, pH 7.0, containing 0.5% (w/v) PEG8000. Crystallization was performed under Al's oil (D'Arcy et al. 1996) (1:1 ratio of paraffin and silicone liquid oils) for 5–14 days at 20°C. Data were collected on beam line ID14eh2 at the ESRF synchrotron (Grenoble, France). Crystals were cryo-protected with a 25% ethylene glycol solution, mounted, and flash cooled to 100 K. X-ray diffraction images were processed using HKL2000 and scaled with SCALEPACK (Otwinowski et al. 1997). The structure was solved using the molecular replacement method based on PDB 2J25 (Brumshtein et al. 2006) and refined with Refmac5 (Murshudov et al. 1997). During the course of refinement, the electron density map showed significant improvement, and putative sugars could be seen adjacent to N19 and N146 for molecule A, and adjacent to N19 for molecule B. Table I summarizes data collection and processing. Structures and structure factors were deposited in the PDB (code 2WKI).

Enzyme kinetics and specific activity

The novel enzymatic activity assay described below measures the ability of GlcCerase to release glucose from GlcCer obtained from Gaucher spleen (Matreya LLC, PA, Cat. no. 1057). Velaglycerase α (drug substance lot EP06-003) and imiglycerase (commercial product lot C7036C01) were assayed. The released glucose was quantified by anion-exchange chromatography equipped with a pulsed amperometric detector. The appropriate amount of GlcCer in chloroform/methanol (2:1, v/v) was dried by a SpeedVac in the presence of 0.2 M taurocholic acid in methanol and 20% (v/v) oleic acid in chloroform/methanol (2:1). The dried pellet was reconstituted in the 0.1 M citrate/0.2 M phosphate buffer (pH 5.0) and diluted to the desired concentrations. Enzyme samples were diluted to a concentration of 0.2 ng/ μ L with the dilution buffer (50 mM sodium citrate, pH 6.0 with 0.75 mg/mL BSA) and 2 ng of enzyme was incubated for 30 min at 37°C with serial dilutions of GlcCer in a 110 μ L reaction volume. The reaction was stopped by heat denaturing samples at 100°C for 5 min. Sample manipulations were internally controlled by adding 100 μ L of a galactosamine (GalN) solution to the reaction mixture. Dionex OnGuard II RP cartridges were used to remove the detergent and lipid. The analysis was carried out on a Dionex high-performance anion-exchange chromatography device, coupled with a pulsed amperometric detection apparatus (HPAE-PAD), using a CarboPac PA-10 analytical column equipped with a CarboPac PA-10 guard column. An isocratic flow of 6 mM NaOH at 0.25 mL/min for 25 min was used to separate monosaccharides (Glc and GalN). The amount of glucose (Glc) was calculated from linear regression analysis of GalN and Glc standards in the range of 10–480 pmol per injection. The assay was carried out in a range of substrate concentrations of 5–150 μ M, and obeyed Michaelis-Menten kinetics, thus permitting assignment of K_m and V_{max} values.

Site-specific characterization of glycans

Velaglycerase α (drug substance lot EP06-003, Shire Human Genetic Therapies, Hampshire, UK) and imiglycerase (commercial product lot HA163BL) were prepared for enzymatic digestion by reductive denaturation with DTT, followed by and

cysteine alkylation with iodoacetic acid. Alkylated samples were digested first with the endoproteinase Lys-C (Roche Diagnostics GmbH, Mannheim, Germany) (1:42 enzyme to substrate ratio, w/w, for 6 h at 37°C), followed by digestion with endoproteinase Glu-C (1:25 enzyme to substrate ratio, w/w, for 16 h at room temperature). Digested samples were analyzed by peptide mass mapping using reversed phase chromatography with in-line UV (214 nm) and electrospray ionization with mass spectrometric detection (LC-ESI-MS). By comparing the peptide maps before and after glycan release using *N*-glycosidase F (New England Biolabs, Ipswich, MA), the five potential glycosylation sites were identified. The glycan mass was calculated by subtracting the expected peptide mass from the observed glycopeptide masses. Using software to match the observed glycan masses with potential monosaccharide compositions, glycan compositions for each site were determined. To verify monosaccharide compositions, treatments (according to manufacturer's recommendations) with neuraminidase (Roche Diagnostics GmbH, Germany), alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany), and α -mannosidase (Glyko, Inc., Hayward, CA) were used to verify the presence of sialic acid, phosphate, and alpha-linked mannose, respectively. MS/MS fragmentation analysis was used to verify glycan phosphorylation.

Glycan map analysis

The procedure involves heat denaturation of the protein at 100°C for 3–4 min in the presence of 0.5% SDS, followed by enzymatic release of glycans with *N*-glycosidase F (Prozyme, San Leandro, CA). Velaglycerase alfa (drug substance lot EP06-001, Shire Human Genetic Therapies) was incubated with *N*-glycosidase F (30 mU/3 μ L) for 4–6 h at 37°C with 0.9% NP40, followed by a second addition of *N*-glycosidase F, and an additional 17–19 h incubation at 37°C. Analysis of the released glycans was performed by HPAE-PAD, using a CarboPac PA-1 analytical column equipped with a CarboPac PA-1 guard column (Dionex, Sunnyvale, CA). Glycans were applied to the column in 12 mM sodium acetate/100 mM NaOH, followed by elution with a 12–300 mM sodium acetate gradient (6.4 mM/min) in 100 mM NaOH in 45 min. Using a flow rate of 1 mL/min and the column at ambient room temperature, glycans elute in the order of increasing negative charge.

Cellular internalization

Human U937 cells were cultured in growth media containing RPMI 1640 with 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, and 10% FBS. Treatment with phorbol myristate acetate (PMA) for 3 days was used to induce differentiation into macrophages (Amento et al. 1984). The U937-derived macrophages were seeded into 96-well microtiter plates at 50,000 cells per well in growth medium, and allowed to adhere to the plates for 48 h. Seeded macrophages were incubated for 3 h with equimolar preparations of velaglycerase alfa (drug substance lot FEC06-003, Shire Human Genetic Therapies) or imiglucerase (Cerezyme®; commercial product lot C7036C01, Genzyme, Cambridge, MA) at pH 7.5, in growth medium containing RPMI 1640 devoid of phosphate, 0.1% BSA, 10 mM HEPES, pH 7.5, 2 mM L-glutamine, 1 mM DTT, and 10 mM CaCl₂. In all assays, the cells were treated with GlcCer for a 3-h duration which was previously determined to be in the

linear range of internalization. For dose response curves utilized to demonstrate mannose-receptor specificity, 10 mg/mL mannan was used to antagonize the receptor. After a series of wash steps (wash buffer: 0.05 M Tris, 0.138 M NaCl, 0.0027 M KCl, with 0.05% Tween 20, 0.5% BSA, pH 8.0), the cells were lysed (lysis buffer: 10 mM Tris pH 8.0, 0.5% NP40, 0.2% deoxycholate, Complete Mini Protease Inhibitor Cocktail Tablets in EASYpacks and PhosSTOP Phosphatase Inhibitor Cocktail Tablets in EASYpacks, Roche Applied Science), and the internalized GlcCer was quantified by an assay employing the synthetic substrate, 4-methylumbelliferyl- β -D-glucopyranoside (4-MU-glc), which releases a fluorescent product upon cleavage. The protein content in the well was determined (BCA method according to the manufacturer's protocol) and was used to normalize the assay signal to total protein from each sample. The assay signal for the GlcCer samples was tested in vitro to determine the extent of activity or signal disparity between the two drugs, and there was no difference in activity (data not shown). For these assays, 2-fold serial dilutions of velaglycerase alfa and imiglucerase (starting at 30 nM enzyme) were made in the assay lysis buffer and tested using the 4-MU-glc enzymatic activity assay. Plates were read with a Perkin Elmer Envision multi-label plate reader.

Funding

Shire Human Genetic Therapies, Inc.

Acknowledgements

J.L. Sussman is the Morton and Gladys Pickman Professor of Structural Biology, and A.H. Futerman is the Joseph Meyerhoff Professor of Biochemistry at the Weizmann Institute of Science. The contribution of Meng Wu, for technical assistance is gratefully acknowledged. We are grateful to Dr. Hilary Voet (Faculty of Agriculture, The Hebrew University, Rehovot) for invaluable discussions concerning the statistical analysis of the choice of space groups.

Conflict of interest statement

None declared.

Abbreviations

CHO, Chinese hamster ovary; ERT, enzyme replacement therapy; GA-GCB, velaglycerase alfa; GlcCer, glucosylceramide; GlcCer, acid- β -glucosidase; M6P, mannose-6-phosphate; prGCD, GlcCer expressed in transgenic carrot cells; RMSD, root mean square deviation.

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In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment G

Letter from FDA to Transkaryotic Therapies, Inc., dated January 12, 2004,
providing the IND number and showing the date of receipt by FDA of the IND



DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Rockville, MD 20857

JAN 19 2004

IND 61,220

Transkaryotic Therapies, Inc.
Attn: Suzanne L. Bruhn, Ph.D.
Vice President, Regulatory Affairs
700 Main Street
Cambridge, MA 02139

Dear Dr. Bruhn:

We acknowledge receipt of your Investigational New Drug Application (IND) submitted under section 505(i) of the Federal Food, Drug, and Cosmetic Act. Please note the following identifying data:

IND Number Assigned: 61,220

Sponsor: Transkaryotic Therapies, Inc.

Name of Drug: Gene-Activated[®] Glucocerebrosidase (GA-GCB, DRX008A)

Date of Submission: December 30, 2003

Date of Receipt: December 31, 2003

Studies in humans may not be initiated until 30 days after the date of receipt shown above. If, on or before January 30, 2004, we identify deficiencies in the IND that require correction before human studies begin or that require restriction of human studies, we will notify you immediately that (1) clinical studies may not be initiated under this IND ("clinical hold") or that (2) certain restrictions apply to clinical studies under this IND ("partial clinical hold"). In the event of such notification, you must not initiate or you must restrict such studies until you have submitted information to correct the deficiencies, and we have notified you that the information you submitted is satisfactory.

It has not been our policy to object to a sponsor, upon receipt of this acknowledgement letter, either obtaining supplies of the investigational drug or shipping it to investigators listed in the IND. However, if the drug is shipped to investigators, they should be reminded that studies may not begin under the IND until 30 days after the IND receipt date or later if the IND is placed on clinical hold.

As sponsor of this IND, you are responsible for compliance with the Federal Food, Drug, and Cosmetic Act and the implementing regulations (Title 21 of the Code of Federal Regulations). Those responsibilities include (1) reporting any unexpected fatal or life-threatening adverse experience associated with use of the drug by telephone or fax no later than 7 calendar days after initial receipt of the information [21 CFR 312.32(c)(2)]; (2) reporting any adverse experience associated with use of the drug that is both serious and unexpected in writing no later than 15 calendar days after initial receipt of the information [21 CFR 312.32(c)(1)]; and (3) submitting annual progress reports [21 CFR 312.33].

Please forward all future communications concerning this IND in triplicate, identified by the above IND number, to the following address:

U.S. Postal Service/Courier/Overnight Mail:
Food and Drug Administration
Center for Drug Evaluation and Research
Division of Metabolic and Endocrine Drug Products, HFD-510
Attention: Fishers Document Room, 8B-45
5600 Fishers Lane
Rockville, Maryland 20857

If you have any questions, call me at (301) 827-6416.

Sincerely,

{See appended electronic signature page}

Patricia Madara
Regulatory Project Manager
Division of Metabolic & Endocrine Drug Products
Office of Drug Evaluation II
Center for Drug Evaluation and Research

**This is a representation of an electronic record that was signed electronically and
this page is the manifestation of the electronic signature.**

/s/

Patricia Madara
1/12/04 02:28:37 PM

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNANOSE PROTEINS AND METHODS OF MAKING HIGH
MANNANOSE PROTEINS

Attachment G1

A written record of the discussion that occurred on January 28, 2004 regarding
modification of the protocol

**FDA CONTACT REPORT
DRX008A**

Contact:	Pat Madara, Metabolic and Endocrinologic Group
	Phone: 301-827-6416
Date:	28 Jan 2004
Time:	10:30

TKT Participants: Steve Schmitz (SS)

Executive Summary:

- Pat Madara telephoned to say that Dr. Pariser, the medical reviewer for the IND submission, informed her that she had not received the protocol amendment, which incorporated the changes discussed at the Pre-IND meeting.
- If she did not receive the amendment, the program would be placed on clinical hold.
- I informed her that we were prepared to send out the amendment within the next 1-2 days.

Action Item:

- Send out the protocol amendment to Pat Madara.

Signature:



Date:

28 Jan 2004

Copy List: W. Aliski
R. Fram

RA Archives (original)
RA Chronology

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment G2

Letter from Transkaryotic Therapies, Inc. to FDA dated March 11, 2004,
concerning amendment of protocol

11 March 2004

Patricia Madara
Regulatory Project Manager
Division of Metabolic and Endocrine Drug Products
Office of Drug Evaluation II
Center for Drug Evaluation and Research
5600 Fishers Lane
Rockville, MD 20857

RE: IND# 61,220
Amendment 2.0, Clinical Protocol TKT025
Serial No. 002

Product Name: Mannose-terminated, Gene-Activated[®] Glucocerebrosidase,
(GA-GCB, DRX008A)

Dear Ms. Madara:

Please find enclosed a copy of Amendment 2.0 to Clinical Protocol TKT025 (Attachment 1), and a document entitled, "Listing of Changes in Amendment No. 2 to TKT Clinical Protocol No. TKT025 (Attachment 2).

The changes include:

- 1) Addition of a MRI of the lumbar spine. Originally, the MRI evaluation examined only the femora and abdomen. However, in order to enhance the assessment of bone marrow involvement by the Bone Marrow Burden Score (see below), an MRI of the lumbar spine is required.
- 2) The addition of an exploratory clinical activity variable, the bone marrow burden score. This score is obtained by evaluating MRI images of both the axial (lumbar spine) and peripheral (femora).
- 3) Clarification of timing of vital sign determination
- 4) Itemization, by "bulleting", in the Schedule of Events, to specify electrocardiogram testing at Weeks 21 and 33.
- 5) Updating of the Informed Consent to describe the additional tests mentioned above. In addition, a statement regarding the potential eligibility of a patient who declined to enter the study, to receive approved therapy (i.e., imiglucerase), was deleted. TKT believed that the statement, as written in the Amendment 1.0, had potential to be misleading to patients. We were concerned that a patient could possibly interpret the previous wording to mean that, in the event that he declined to participate in the study, the Sponsor would provide imiglucerase, a currently approved therapy for Gaucher disease.

If you have any questions or comments, please telephone me at 617-613-4364. Thank you for your consideration.

Sincerely,

A handwritten signature in black ink, appearing to read "Stephen M. Schmitz". The signature is fluid and cursive, with the first name "Stephen" and last name "Schmitz" clearly distinguishable.

Stephen M. Schmitz, M.D., M.P.H.
Director, Safety and Regulatory Affairs
Transkaryotic Therapies, Inc.

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment G3

FDA communication to Transkaryotic Therapies, Inc. dated May 20, 2004,
concerning amendment to protocol

AGENCY CORRESPONDENCE

GCB

Contact(s)	Pat Madara, Project Manager Div. of Metabolic and Endocrinologic Drug Products/CDER/FDA 301-827-6416
Date	20 May 2004
Time	1400 and 1415 (Hours and Minutes in Military Time)

Agency Participants

Pat Madara, Project Manager (PM)

Re: IND 61,220 – DRX008A (glucocerebrosidase, GA-GCB)

TKT Participants

Suzanne L. Bruhn, VP, Reg. Affairs (SB)

Alyssa Sonntag, Project Manager, Reg. Affairs (AS)

Executive Summary

- FDA has no comments on the blinding procedure to be implemented for the analysis of liver and spleen volumes from MRI scans taken for Study TKT025. TKT will make no changes to the current version of the blinding procedure (dated 7 April 2004).

Summary

At 1400, SB called PM to inquire on the status of FDA's review of the blinding procedure for analysis of liver and spleen volumes from the MRI scans taken for Study TKT025, which was submitted to the IND in Serial 003 on 7 April 2004. PM stated that the Medical Officer's review of this submission had been completed but she would need to confirm if there were any comments to relay to the sponsor from that review. PM stated that she would contact us soon with this information, but we should call her again after 2 weeks if we had not heard from her. PM will be out of the office during the last week of May and the first week of June.

PM called back at 1415 and informed SB that she had pulled the Medical Officer's review and that it was "safe to proceed [with the blinding procedure] as amended." Therefore, TKT will make no changes to the current version of the blinding procedure (dated 7 April 2004).

Serial Submissions Discussed

Serial 003, submitted to IND 61,220 on 7 April 2004

Action Item(s)

- None

Signature Alyssa Forintez Date 24 May 2004

Copy List Regulatory Chronology (Original w/ signature)

S. Bruhn

R. Fram

S. Zildjian

N. Wyant

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment H

Letter from FDA to Shire Human Genetic Therapies, Inc. indicating the date the
IND was put on clinical hold

REGULATORY AFFAIRS
RECEIVED

NOV 28 2006

FAX

SHIRE HGT



**FOOD AND DRUG ADMINISTRATION
DIVISION OF GASTROENTEROLOGY PRODUCTS**
Center for Drug Evaluation and Research, HFD-180
10903 New Hampshire Ave, Silver Spring, MD 20993-0002

To: Nikhil S. Mehta, Ph.D.

From: Ryan Barraco

Fax: 617-613-4444

Fax: 301-796-9905

Phone: 617-613-4531

Phone: 301-796-0846

Pages, including cover sheet: 8

Date: November 28, 2006

Re: IND 61,220 for GA-GCB – Full Clinical Hold Letter

Comments:

Courtesy Fax

THIS DOCUMENT IS INTENDED ONLY FOR THE USE OF THE PARTY TO WHOM IT IS ADDRESSED AND MAY CONTAIN INFORMATION THAT IS PRIVILEGED, CONFIDENTIAL AND PROTECTED FROM DISCLOSURE UNDER APPLICABLE LAW. If you are not the addressee, or a person authorized to deliver the document to the addressee, you are hereby notified that any review, disclosure, dissemination or other action based on the content of the communication is not authorized. If you have received this document in error, please immediately notify us by telephone and return it to us at the above address by mail. Thank you.

**DEPARTMENT OF HEALTH & HUMAN SERVICES**

Public Health Service

Food and Drug Administration
Rockville, MD 20857

IND 61,220

FULL CLINICAL HOLD

Shire Human Genetic Therapies
Attention: Nikhil Mehta, Ph.D.
Vice President, Global Regulatory Affairs
700 Main Street
Cambridge, MA 02139

Dear Dr. Mehta:

Please refer to your Investigational New Drug Application (IND) submitted December 30, 2003, received December 31, 2003, under section 505(i) of the Federal Food, Drug, and Cosmetic Act for Gene Activated® Glucocerebrosidase (GA-GCB).

We also refer to your amendment dated August 3, 2006 (serial # 035), and to the November 20, 2006, telephone conversation between you and our Division, in which you were notified that your IND is on clinical hold and any proposed studies may not be initiated. The following are the specific deficiencies [21 CFR 312.42(b)] and the information needed to resolve these deficiencies.

Insufficient information to assess risks to human subjects [21 CFR 312.42(b)(2)(ii)].

Clinical Hold Deficiencies

The chemistry, manufacturing, and controls (CMC) amendment dated August 3, 2006, did not demonstrate that the two manufacturing processes for Gene Activated® Glucocerebrosidase (GA-GCB) yield drug substances (DS) with comparable physicochemical characteristics. The comparability data provided in this amendment revealed that there were the following physicochemical differences between DS produced by the two processes:

- a. For the glycan mapping, the total percentage of Group 1 carbohydrates is different between the two DS, and it appears that relative proportions of individual peaks within Group 1 are different in the two DS.
- b. Although the predominant peaks C, D, and E in IEX-HPLC constitute more than 75% of the total peak area and meet the acceptance criteria, there is a significant shift in the proportion of each peak, which does not appear to be due to assay variability.
- c. The pattern of bands detected by IEF gels Coomassie stained is different in DS manufactured with the serum containing process (E303-006) versus DS

IND 61,220
Page 2

manufactured with the animal-free process (EP06-003). At least one additional acidic species is present in E303-006.

As you intend to use the DS manufactured using the new manufacturing process in your proposed Phase 3 clinical study, and as this DS has not been evaluated in pre-clinical or clinical studies, insufficient information exists with this DS to assess the risks to human subjects for the proposed Phase 3 clinical investigation.

Information needed to resolve clinical hold deficiencies

1. You must demonstrate the comparability of DS by the two manufacturing processes as set forth in the "Guidance for Industry Q5E Comparability of Biotechnological/Biological Products Subject to Changes in their Manufacturing Process."
2. Alternatively, you may propose to perform your Phase 3 clinical study using the same DS administered in the completed Phase 1/2 study and in the pre-clinical testing conducted in support of this Phase 1/2 study.
3. Alternatively, you may propose to repeat pre-clinical and clinical studies with DS manufactured using the new manufacturing method, which are needed to support the proposed Phase 3 clinical study. These studies are to include:
 - a. A head-to-head comparison of the two DS in *in vitro* and *in vivo* pharmacology studies to demonstrate comparability of the two DS on the primary pharmacological effect of GA-GCB.
 - b. A head-to-head tissue distribution comparison of the two DS in Sprague Dawley rats of both sexes.
 - c. A clinical study to assess the pharmacokinetics, pharmacodynamics, and preliminary safety of GA-GCB administration.

Until you have submitted the required information, and we notify you that you may initiate the trial, you may not legally conduct the identified clinical study under this IND.

Please identify your response to the clinical hold issues as a "CLINICAL HOLD COMPLETE RESPONSE." To facilitate a response to your submission, submit this information in triplicate to the IND. In addition, send a copy of the cover letter to Ryan Barraco.

Following receipt of your complete response to these issues, we will notify you of our decision within 30 days.

In addition, we have the following recommendations and requests that are important for product development, but are not clinical hold issues at this time. Your responses to any non-hold issues should be addressed in a separate amendment to the IND.

IND 61,220
Page 3

CMC

Regarding characterization and release testing:

1. You are currently measuring enzymatic activity using a surrogate substrate. In order to properly characterize DS and drug product (DP), measurements of the K_m and k_{cat} kinetic parameters using a physiologically relevant substrate should be performed.
2. Routine DS and DP release testing should also include (i) measurements of the K_m and k_{cat} kinetic parameters using a physiologically relevant substrate, and (ii) a quantitative assessment of receptor binding and uptake by macrophages. Please note that glycan mapping is not considered to be a potency assay from a regulatory perspective.
3. An in-depth characterization of the glycan structures in GA-GCB, with information on branching and size of the glycan chains, mannose-6P content and residual content of NANA should be performed. Adequate assays that allow for control of carbohydrate content and structure should be included in release testing.
4. The area for each peak identified in groups 1, 2 and 3 of the glycan mapping assay should be specified in your release testing.
5. N-terminal sequencing and Western blotting for identity, and Ion Exchange Chromatography for purity were not performed at release testing for the clinical lots of DS. These tests assess critical product attributes and should be maintained as release tests.
6. Your current DS acceptance criteria for RP-HPLC and SE-HPLC are $\geq 94\%$ of the main peak area. From the results of batch analysis, it appears that on average, RP-HPLC purity is about 98% and SE-HPLC is about 97%. Acceptance criteria should be established based on process capability and manufacturing experience, and we recommend establishing more stringent specifications.
7. It appears that the new process generates a DS with a higher percent of aggregates (by comparison of batch analysis results). These aggregates should be characterized, and an orthogonal method to detect aggregates should be used to validate SE-HPLC.
8. It is not clear whether studies were performed to detect impurities that could arise from the DP manufacturing process. The increase in aggregation of GA-GCB during DP manufacturing should be documented, and procedures implemented to minimize aggregate formation.

IND 61,220
Page 4

9. You have significantly changed DP acceptance criteria for SE-HPLC and RP-HPLC to $\geq 92\%$ main peak area. Although you justify these acceptance criteria based on limited manufacturing experience, they appear to allow for excessive amounts of aggregates and impurities in the DP. Acceptance criteria should be established based on process capability and manufacturing experience, and we recommend establishing more stringent specifications.

Regarding the manufacturing process:

10. You provided flow charts for the manufacturing process that include in-process controls. It appears that the only in-process control in the purification step is protein recovery. We recommend inclusion of additional in-process tests that could provide information on purity such as, but not limited to, SDS-PAGE reducing and non-reducing.
11. You stated that impurities derived from the culture medium, such as plant hydrolysates, kifunensine and DTT will be removed during the purification process. You should provide supportive data for the above claims. Presence of impurities should be assessed and specified at critical steps, and in lot release. Alternatively, removal of the process-related impurities must be validated.
12. Acceptance criteria for HCP content should be modified to reflect the actual capability of the process to remove these impurities. Currently, acceptance criterion is <200 ng/mg, and the actual results range from 7 to 23 ng/mg.
13. You state that antibodies have been raised against protein lysates from cells growing in serum-free and serum-containing medium. Please clarify which antibodies have been used to develop the ELISA and Western blotting assays.

Regarding stability:

14. It is important to demonstrate that critical drug potency parameters are not altered at the indicated storage temperature. Please include the following assays in your stability testing program for DS and DP:
 - a. Macrophage uptake assay and receptor binding assay.
 - b. Measurements of the K_m and k_{cat} kinetic parameters.
 - c. All assays should be evaluated for stability indicating potential.
15. Please refer to ICH Q5C for guidance on stability studies for biotechnology products.

IND 61,220
Page 5

Regarding cell banks:

16. You provided a stability program for MCB and WCB of up to four years. Viability and growth should be assessed at later times as well, to ensure that a constant supply of starting material is reproducibly available.
17. Please submit data regarding viral clearance by filtration for review as soon as they are available.

Clinical/Statistical

Regarding your proposed Phase 3 clinical protocol:

18. Your protocol contains a large number of secondary endpoints. If you intend to use any of these secondary endpoints to support the indication for the treatment of type 1 Gaucher disease with GA-GCB, you will need to include in your statistical analysis plan a proposal for evaluating these endpoints in a statistically rigorous manner that accounts for multiplicity.
19. Please provide a rationale for performing pharmacokinetic sampling to evaluate the multiple-dose pharmacokinetics of GA-GCB at Week 37.
20. Your protocol excludes from study participation patients who are anti-imiglucerase IgG antibody positive. As stated at the End of Phase 2 (EOP2) meeting on January 11, 2006, it is likely that at least some of the patients in clinical practice who transition from Cerezyme® to GA-GCB will be IgG anti-imiglucerase antibody positive. We recommend that the inclusion criteria be broadened to include type 1 Gaucher Disease patients regardless of imiglucerase-antibody status, as inclusion of these patients would more accurately represent the expected clinical use of GA-GCB, and would support the use of GA-GCB in a broader patient population.
21. The stopping rules for your study (in protocol section 9.4) state that "If any patient experiences a life-threatening (Grade 4) serious adverse event (SAE), or death occurs that is considered possibly or probably related to the study drug, the decision to stop the study requires agreement by the Shire HGT Medical Monitor, the Investigator, and the IRB/IEC." Please revise the stopping rules for the study based on specific safety criteria, rather than on the subjective assessment of events by study personnel.
22. The procedures for Serious Adverse Event (SAE) reporting (in protocol section 9.3.1) state that "Any SAE that occurs after administration of the first dose of GA-GCB must be reported in the event of a severe, possibly or probably related AE or SAE..." Please revise the SAE reporting procedures in your protocol to more accurately reflect the requirements under 21 CFR 312.32(c)(1) and (2), whereby "The sponsor shall notify FDA and all participating investigators in a written IND safety report of: (A) Any adverse experience associated with the use of the drug that is both serious and

IND 61,220
Page 6

unexpected..." and "The sponsor shall also notify FDA by telephone or by facsimile transmission of any unexpected fatal or life-threatening experience associated with the use of the drug... in no event later than 7 calendar days..." Please note that this requirement does not include a subjective assessment (possibly or probably related) of the event.

23. Please revise your protocol so that all pediatric patients participating in the study are to undergo assessments of growth at regular intervals in the study. Assessments of growth including, at minimum, assessments of height and weight, are to be obtained in a standardized manner that are to be delineated in the study protocol (e.g., height measured via a calibrated stadiometer, and the final measurement taken as an average of three measurements).
24. Your sample Informed Consent form states (on page 5, paragraph 7) that children as young as two years of age will be undergoing magnetic resonance imaging (MRI) testing. In young children, sedation is often required for MRI testing. We recommend that you revise your sample Informed Consent form to note the possible need for sedation in pediatric patients for MRI testing, and an explanation of the risks of sedation in these patients.

Please cite the IND number listed above at the top of the first page of any communications concerning this application. Send all submissions, electronic or paper, including those sent by overnight mail or courier, to the following address:

Food and Drug Administration
Center for Drug Evaluation and Research
Division of Gastroenterology
5901-B Ammendale Road
Beltsville, MD 20705-1266

If you have any questions, call Ryan Barraco, Regulatory Project Manager, at (301) 796-0846.

Sincerely,

{See appended electronic signature page}

Brian E. Harvey, M.D., Ph.D.
Director
Division of Gastroenterology Products
Office of Drug Evaluation III
Center for Drug Evaluation and Research

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this page is the manifestation of the electronic signature.**

/s/

Brian Harvey

11/27/2006 04:00:35 PM

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment I

Letter from FDA to Shire Human Genetic Therapies, Inc., dated December 21,
2006, removing the clinical hold and indicating that the protocol can be initiated



DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Rockville, MD 20857

IND 61,220

Shire Human Genetic Therapies
Attention: Nikhil Mehta, Ph.D.
Vice President, Global Regulatory Affairs
700 Main Street
Cambridge, MA 02139

REGULATORY AFFAIRS
RECEIVED

REGULATORY AFFAIRS
RECEIVED

JAN 5 2007

SHIRE HGT

SHIRE HGT

Dear Dr. Mehta:

Please refer to your Investigational New Drug Application (IND) submitted December 30, 2003, under section 505(i) of the Federal Food, Drug, and Cosmetic Act for Gene Activated Glucocerebrosidase (GA-GCB).

We also refer to your amendment dated November 30, 2006 (serial # 041), which provided a complete response to our December 7, 2006, letter which cited the reasons for placing Protocol TKT032, titled "A Multi-center, Randomized, Double-Blind, Parallel Group, Two-Dose Study of Gene-Activated™ Human Glucocerebrosidase (GAGCB) Enzyme Replacement Therapy in Patients with Type I Gaucher Disease," on clinical hold and the information needed to resolve the clinical hold issues.

We have completed the review of your submission, and have concluded that the above protocol may be initiated.

We have the following comments, however, regarding your clinical development program:

A direct comparison of IEX-HPLC and glycan mapping data for drug substance (DS) lots E303-005, E303-006, E303-007, and EP06-003 indicate that physico-chemical differences exist between the DS manufactured with different processes that were used in clinical trials. However, these differences do not appear to pose a serious safety risk to human subjects, and clinical trials with the Animal Free (AF) DS appear to be safe to proceed at this time. Nevertheless, in view of these differences, please be aware that you might not be able to use the Phase 1 clinical data generated using the serum-containing DS to support a future marketing application for GA-GCB.

As sponsor of this IND, you are responsible for compliance with the Federal Food, Drug, and Cosmetic Act and the implementing regulations (Title 21 of the Code of Federal Regulations). Those responsibilities include (1) reporting any unexpected fatal or life-threatening adverse experience associated with use of the drug by telephone or fax no later than 7 calendar days after initial receipt of the information [21 CFR 312.32(c)(2)]; (2) reporting any adverse experience associated with use of the drug that is both serious and unexpected in writing no later than 15 calendar days after initial receipt of the information [21 CFR 312.32(c)(1)]; and (3) submitting annual progress reports [21 CFR 312.33].

IND 61,220
Page 2

If you have any questions, call Ryan Barraco, Regulatory Project Manager, at (301) 796-0846.

Sincerely,

{See appended electronic signature page}

Brian E. Harvey, M.D., Ph.D.
Director
Division of Gastroenterology Products
Office of Drug Evaluation III
Center for Drug Evaluation and Research

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SHIRE HGT

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this page is the manifestation of the electronic signature.**

/s/

Brian Harvey

12/21/2006 05:11:30 PM

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

**Assignee: Shire Human Genetic Therapies,
Inc.**

**Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS**

Attachment J

**Letter from FDA to Shire Human Genetic Therapies, Inc., dated September 14,
2009, acknowledging receipt of the final submission of the NDA**



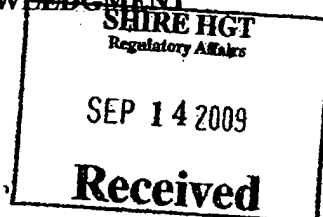
DEPARTMENT OF HEALTH AND HUMAN SERVICES

Food and Drug Administration
Silver Spring MD 20993

NDA 22575

Shire Human Genetic Therapies, Inc.
Attention: Nikhil Mehta, Ph.D.
Vice President, Global Regulatory Affairs
700 Main Street
Cambridge, MA 02139

NDA ACKNOWLEDGMENT



Dear Dr. Mehta:

We have received your new drug application (NDA) submitted under section 505(b) of the Federal Food, Drug, and Cosmetic Act (FDCA) for the following:

Name of Drug Product: TRADENAME (velaglucerase alfa)

Date of Application: August 31, 2009

Date of Receipt: August 31, 2009

Our Reference Number: NDA 22575

Unless we notify you within 60 days of the receipt date that the application is not sufficiently complete to permit a substantive review, we will file the application on October 30, 2009, in accordance with 21 CFR 314.101(a).

If you have not already done so, promptly submit the content of labeling [21 CFR 314.50(l)(1)(i)] in structured product labeling (SPL) format as described at <http://www.fda.gov/oc/datacouncil/spl.html>. Failure to submit the content of labeling in SPL format may result in a refusal-to-file action under 21 CFR 314.101(d)(3). The content of labeling must conform to the content and format requirements of revised 21 CFR 201.56-57.

The NDA number provided above should be cited at the top of the first page of all submissions to this application. Send all submissions, electronic or paper, including those sent by overnight mail or courier, to the following address:

Food and Drug Administration
Center for Drug Evaluation and Research
Division of Gastroenterology Products
5901-B Ammendale Road
Beltsville, MD 20705-1266

All regulatory documents submitted in paper should be three-hole punched on the left side of the page and bound. The left margin should be at least three-fourths of an inch to assure text is not obscured in the fastened area. Standard paper size (8-1/2 by 11 inches) should be used; however, it may occasionally be necessary to use individual pages larger than standard paper size. Non-standard, large pages should be folded and mounted to allow the page to be opened for review without disassembling the jacket and refolded without damage when the volume is shelved. Shipping unbound documents may result in the loss of portions of the submission or an unnecessary delay in processing which could have an adverse impact on the review of the submission. For additional information, please see <http://www.fda.gov/cder/ddms/binders.htm>.

If you have any questions, call me at (301) 796-0069.

Sincerely,

{See appended electronic signature page}

R. Wesley Ishihara
Regulatory Health Project Manager
Division of Gastroenterology Products
Office of Drug Evaluation III
Center for Drug Evaluation and Research

Application
Type/Number

Submission
Type/Number

Submitter Name

Product Name

NDA-22575

ORIG-1

SHIRE HGT INC.

VELAGLUCERASE ALFA

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/s/

RICHARD W ISHIHARA
09/14/2009

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re U.S. Patent No.: 7,138,262 B1

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

CERTIFICATE OF EXPRESS MAILING UNDER 37 C.F.R. §1.10

The undersigned hereby certifies that this document was deposited with the U.S. Postal Service on April 22, 2010 for express mailing in accordance with §1.6(a)(2).


Laurie Butler Lawrence, Reg. No. 46,593

Mail Stop Hatch-Waxman PTE

Commissioner for Patents

P.O. Box 1450

Alexandria, VA 22313-1450

APPLICATION FOR EXTENSION OF PATENT TERM UNDER 35 U.S.C. § 156

Applicant, Shire Human Genetic Therapies, Inc. represents that it is the Assignee of the entire interest in and to United States Patent No. 7,138,262 B1 granted to Shire Human Genetic Therapies, Inc. on the 21st day of November 2006, for "High Mannose Proteins and Methods of Making High Mannose Proteins" by virtue of an assignment from Peter Francis Daniel to Transkaryotic Therapies, Inc., recorded in the U.S. Patent and Trademark Office at Reel 011662, Frame 0815, on March 28, 2001, and from Transkaryotic Therapies, Inc. to Shire Human Genetic Therapies, Inc., recorded in the U.S. Patent and Trademark Office at Reel 018224, Frame 0390, on August 31, 2006.

By the Power of Attorney enclosed herein (Attachment A), Applicant has appointed several individual attorneys, including Laurie Butler Lawrence, as attorneys for Shire Human Genetic Therapies, Inc. with regard to this application for extension of the term of U.S. Patent No. 7,138,262 B1 and to transact all business in the U.S. Patent and Trademark Office in connection therewith.

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Transkaryotic Therapies, Inc. became Shire Human Genetic Therapies, Inc. on January 17, 2006. Shire Human Genetic Therapies, Inc. is the holder of the regulatory approval granted with respect to the regulatory review period relied on herein.

Information Required Under 37 C.F.R. § 1.740

Applicant hereby submits this application for extension of the patent term under 35 U.S.C. § 156 by providing the following information required by the rules promulgated by the U.S. Patent and Trademark Office (37 C.F.R. § 1.740). For the convenience of the Patent and Trademark Office, the information contained in this application will be presented herein in a format which follows the order of the requirements of Section 1.740 of Title 37 of the Code of Federal Regulations.

(1) Identification of the Approved Product [1.740(a)(1)]

The approved product is VPRIVTM. The name of the active ingredient in VPRIVTM is velaglucerase alfa for injection. Velaglucerase alfa for injection is a hydrolytic lysosomal glucocerebroside-specific enzyme indicated for long-term replacement therapy (ERT) for pediatric and adult patients with type 1 Gaucher disease. The active ingredient of VPRIVTM is velaglucerase alfa, which is produced by gene activation technology in a human fibroblast cell line. Velaglucerase alfa is a glycoprotein of 497 amino acids; with a molecular weight of approximately 63 kDa. Velaglucerase alfa has the same amino acid sequence as the naturally occurring human enzyme, β -glucocerebrosidase. Velaglucerase alfa contains 5 potential N-linked glycosylation sites; four of these sites are occupied by glycan chains. Velaglucerase alfa is manufactured to contain predominantly high mannose-type N-linked glycan chains. VPRIVTM is supplied as a sterile, preservative free, lyophilized powder in single-use vials. Following reconstitution with Sterile Water for Injection, USP, the solution contains the components listed in Table 3 of the package insert, which is provided in Attachment B (a copy of the

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

package insert is also provided as an enclosure to the NDA approval letter from FDA to Shire Human Genetic Therapies, Inc., dated February 26, 2010 in Attachment C).

(2) Federal Statute Governing Regulatory Approval of the Approved Product [1.740(a)(2)]

The approved product, VPRIVTM, was subject to regulatory review under § 505(i) and §505(b) of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. § 355(i) and § 355(b)).

(3) Date of Approval for Commercial Marketing [1.740(a)(3)]

The approved product, VPRIVTM, received permission for commercial marketing or use under Section 505(b) of the Federal Food, Drug, and Cosmetic Act on February 26, 2010. A copy of the NDA approval letter from FDA to Shire Human Genetic Therapies, Inc., dated February 26, 2010 (with enclosure), is provided as Attachment C.

(4) Identification of Active Ingredient and Certifications Related to Commercial Marketing of Approved Product [1.740(a)(4)]

The only active ingredient in VPRIVTM is velaglucerase alfa for injection which, on information and belief, has not been previously approved for commercial marketing or use under the Public Health Service Act, the Virus-Serum-Toxin Act or the Federal Food, Drug, and Cosmetic Act. A copy of the package insert describing the approved product is attached (Attachment B).

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

(5) Statement Regarding Timeliness of Submission of Patent Term

Extension Request [1.740(a)(5)]

This application for extension of patent term under 35 U.S.C. § 156 is being submitted within the permitted 60-day period pursuant to 37 C.F.R. § 1.720(f). The last day on which this application can be submitted is April 26, 2010.

(6) Complete Identification of the Patent for Which Extension Is Being

Sought [1.740(a)(6)]

The complete identification of the patent for which a term extension is being sought is as follows:

Inventors:	Peter Francis Daniel
Patent No.:	7,138,262 B1
Filing Date:	August 18, 2000
Issue Date:	November 21, 2006
Expiration Date:	August 18, 2020

(7) Copies of the Patent for Which an Extension is Being Sought

[1.740(a)(7)]

A copy of U.S. Patent No.: 7,138,262 B1 is provided as Attachment D.

(8) Copies of Disclaimers, Certificates of Correction, Receipt of

Maintenance Fee Payments, or Reexamination Certificate [1.740(a)(8)]

(a) U.S. Patent No.: 7,138,262 B1 is not subject to a terminal disclaimer.

(b) No certificate of correction has been issued for U.S. Patent No.: 7,138,262 B1.

(c) The first maintenance fee for U.S. Patent No.: 7,138,262 B1 will be due with a payment of the surcharge on May 22, 2010. This maintenance fee has been paid as

In re U.S. Patent No.: 7,138,262 B1
Issued: November 21, 2006
Inventors: Peter Francis Daniel
Assignee: Shire Human Genetic Therapies,
Inc.

Attorney Docket No.: S2071-701019/0013US

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

shown in the copy of the USPTO's on-line record of patent maintenance fee payment for this patent which is attached (Attachment E).

(d) U.S. Patent No.: 7,138,262 B1 has not been the subject of a reexamination proceeding.

(9) Statement Regarding Patent Claims Relative to Approved Product
[1.740(a)(9)]

The following claims of U.S. Patent No. 7,138,262 B1 claim a method of manufacturing the approved product, VPRIVTM: claims 1-4, 12-18, 23, 26-38, 48-54, and 57-61.

(iii) Pursuant to M.P.E.P. § 2753 and 37 C.F.R. § 1.740(a)(9)(iii), the following explanation is provided which demonstrates the manner in which at least one such patent claim reads on the method of manufacturing the approved product, VPRIVTM.

Description of the approved product and the method of manufacturing the same:

Velaglucerase alfa for injection is human β -glucocerebrosidase produced by gene-activation in immortalized human fibroblast HT-1080 cells. Gene activation refers to the introduction of an exogenous promoter into the cell that activates the endogenous human β -glucocerebrosidase gene. The activated gene expresses human β -glucocerebrosidase. β -glucocerebrosidase has 5 potential N-linked glycosylation sites, four of which are occupied by glycan chains in velaglucerase alfa for injection.

Glycosylation of velaglucerase alfa for injection is altered by culturing the cells in the presence of kifunensine, a mannosidase I inhibitor, at 2 μ g/ml. This results in the secretion of human β -glucocerebrosidase containing primarily high-mannose type glycan chains having 6-9 mannose units per glycan chain. The cells are cultured under conditions wherein:

In re U.S. Patent No.: 7,138,262 B1
Issued: November 21, 2006
Inventors: Peter Francis Daniel
Assignee: Shire Human Genetic Therapies,
Inc.

Attorney Docket No.: S2071-701019/0013US

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

the mannosidase inhibitor prevents removal of one or more α 1,2 mannose residue(s) distal to the pentasaccharide core; the removal of one or more mannose residues distal to the pentasaccharide core is prevented on at least two carbohydrate chains of hmGCB; at least 60% of the high mannose glucocerebrosidase (hmGCB) of the preparation have three or more carbohydrate chains in which the removal of one or more mannose residues distal to the pentasaccharide core has been prevented; at least about 60% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues; the mannosidase inhibitor further prevents the removal of one α 1,3 mannose residue distal to the pentasaccharide core; the mannosidase inhibitor further prevents the removal of one α 1,6 mannose residue distal to the pentasaccharide core; the mannosidase inhibitor prevents removal of at least three mannose residues distal to the pentasaccharide core of the precursor oligosaccharide of GCB; at least 60% of the hmGCB of the preparation have one or more carbohydrate chains in which the removal of three or more mannose residues distal to the pentasaccharide core has been prevented; at least 60% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues; and at least about 80% or more of the carbohydrate chains of the hmGCB preparation have six or more mannose residues.

Velaglycerase alfa for injection is harvested from media in which the cells are cultured.

Velaglycerase alfa for injection is secreted as a monomeric glycoprotein of approximately 63 kDa and is composed of 497 amino acids with a sequence identical to the natural human protein. The amino acid sequence of velaglycerase alfa for injection is described in Zimran et al. (2007) *Blood Cells Mol Dis*, 39: 115-118. Velaglycerase alfa for injection contains 5 potential N-linked glycosylation sites; four of these sites are occupied by glycan chains.

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Issued: November 21, 2006
Inventors: Peter Francis Daniel
Assignee: Shire Human Genetic Therapies,
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A summary of the glycan structure, and other aspects of velaglucerase alfa for injection, is provided in Brumshtein et al. (2010) Glycobiology 20(1):24-32 as Attachment F. See, e.g., Table III, therein.

As is discussed below, claims 1-4, 12-18, 23, 26-38, 48-54, and 57-61 of U.S. Patent No. 7,138,262 B1 read on the method of manufacturing the approved product. The claims are set out in the left hand column of the table immediately below. The method of manufacturing the approved product is described in the right hand column and compared with the claim. As is shown, the approved product meets all of the limitations of each of claims 1-4, 12-18, 23, 26-38, 48-54, and 57-61 and claims 1-4, 12-18, 23, 26-38, 48-54, and 57-61 cover the method of manufacturing the approved product, VPRIV™.

<p>1. A method of producing a preparation of high mannose glucocerebrosidase (hmGCB) comprising a carbohydrate chain having at least four mannose residues,</p> <p>comprising: providing a mammalian cell that expresses a human glucocerebrosidase (GCB);</p>	<p>VPRIV™ includes at least two glucocerebrosidase (GCB) proteins that have at least one carbohydrate chain having four or more mannose residues. See, e.g., page 24, column 1 of Attachment F, “the predominant glycan on velaglucerase alfa is a high mannose type, with nine mannose residues”. See also Table III and Figures 7 and 8 of Attachment F. See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>VPRIV™ is produced in a human cell line that expresses human glucocerebrosidase See page 24, column 2 of Attachment F: “we have used gene activation in a well characterized, continuous human cell line to produce gene activated human acid-β-glucocerebrosidase (velaglucerase alfa).” See also the description of the approved product and the method of manufacturing</p>
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<p>contacting the cell with kifunensine; allowing the cell to produce hmGCB; and</p> <p>harvesting the hmGCB from the cell or its culture media, to thereby produce an hmGCB preparation.</p>	<p>the same in this section (9)(iii).</p> <p>The human cell line expressing VPRIVTM is contacted with kifunensine and the cells secrete VPRIV. See page 24, column 2 of Attachment F, "glycosylation of velaglycerase alfa is altered by using kifunensine ... during cell culture, which results in the secretion of a protein containing predominantly high-mannose type glycans" See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>VPRIVTM is harvested from the cell culture to produce a preparation with at least two glucocerebrosidase proteins having at least one carbohydrate chain having four or more mannose residues. See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 1 and the claim covers the method of manufacturing the approved product.</p>
<p>2. The method of claim 1, wherein removal of one or more α1,2 mannose residue(s) distal to the pentasaccharide core is prevented.</p>	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>VPRIVTM includes at least 2 GCB proteins that have one or more mannose residue distal to the pentasaccharide core present. See the description of the approved product and the method of manufacturing the same</p>

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	<p>in this section (9)(iii).</p> <p>Therefore, the method of making VPRIV meets all of the limitations of claim 2 and the claim covers the method of manufacturing the approved product.</p>
<p>3. The method of claim 1, wherein the kifunensine is present at a concentration between about 0.05 to 20.0 µg/ml.</p>	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>Kifunensine is present at a concentration 2.0 µg/ml in the method of making the approved product. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 3 and the claim covers the method of manufacturing the approved product.</p>
<p>4. The method of claim 3, wherein the kifunensine is present at a concentration between about 0.1 to 2.0 µg/ml.</p>	<p>As discussed above for claims 1 and 3, the method of making the approved product meets all of the limitations of the base claims.</p> <p>Kifunensine is present at a concentration of 2.0 µg/ml in the method of making the approved product. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM</p>

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	meets all of the limitations of claim 4 and the claim covers the method of making the approved product.
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12. The method of claim 1, wherein the removal of one or more mannose residues distal to the pentasaccharide core is prevented on at least two carbohydrate chains of hmGCB.	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>Kifunensine is an inhibitor of endoplasmic reticulum mannosidase I and Golgi mannosidase I enzymes (see column 23, lines 48-51 of the '262 patent) that prevents the removal of one or more mannose residues distal to the pentasaccharide core. (see, e.g., Fig. 7 of Attachment F which shows the predominant glycan structure present on the GCB in VPRIVTM). See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 12 and the claim covers the method of manufacturing the approved product.</p>
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13. The method of claim 1, wherein at least 60% of the hmGCB of the preparation have one or more carbohydrate chains in which the removal of one or more mannose residues distal to the pentasaccharide core has been prevented.	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>At least 60% of the hmGCB of the preparation have one or more carbohydrate chains in which the removal of one or more</p>
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	<p>mannose residues distal to the pentasaccharide core has been prevented. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 13 and the claim covers the method of manufacturing the approved product.</p>
14. The method of claim 13, wherein the removal of three or more mannose residues distal to the pentasaccharide core has been prevented.	<p>As discussed above for claims 1 and 13, the method of making the approved product meets all of the limitations of the base claims.</p> <p>The removal of three or more mannose residues distal to the pentasaccharide core has been prevented. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 14 and the claim covers the method of manufacturing the approved product.</p>
15. The method of claim 1, wherein at least about 20% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>At least about 20% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues. See the description of the</p>

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Issued: November 21, 2006
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	<p>approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIV™ meets all of the limitations of claim 15 and the claim covers the method of manufacturing the approved product.</p>
<p>16. The method of claim 15, wherein at least about 40% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.</p>	<p>As discussed above for claims 1 and 15, the method of making the approved product meets all of the limitations of the base claim.</p> <p>At least about 40% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIV™ meets all of the limitations of claim 16 and the claim covers the method of manufacturing the approved product.</p>

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17. The method of claim 16, wherein at least about 60% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.	<p>As discussed above for claims 1, 15 and 16, the method of making the approved product meets all of the limitations of the base claims.</p> <p>At least about 60% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 17 and the claim covers the method of manufacturing the approved product.</p>
18. The method of claim 1, wherein at least about 80% or more of the carbohydrate chains of the hmGCB preparation have six or more mannose residues.	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>At least about 80% or more of the carbohydrate chains of the hmGCB preparation have six or more mannose residues. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 18 and the claim covers the method of manufacturing the approved product.</p>

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<p>23. The method of claim 1, wherein the cell comprises an exogenous regulatory sequence which functions to regulate expression of an endogenous GCB coding sequence.</p>	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>VPRIVTM is produced by a human cell line that comprises an exogenous promoter sequence to regulate expression of an endogenous GCB coding sequence. (see, e.g., page 24, column 2 of Attachment F- “we have used gene activation in a well-characterized, continuous human cell line to produce gene activated human acid-β-glucocerebrosidase (velaglucerase alfa). Gene activation refers to targeted recombination with a promoter that activates the endogenous GlcCerase gene in the selected human cell line.” See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 23 and the claim covers the method of manufacturing the approved product.</p>
<p>26. The method of claim 1, wherein the cell is a human cell.</p>	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>VPRIVTM is produced by a human cell line. See page 24, column 2 of Attachment F – “we have used gene activation in a well characterized, continuous human cell line to produce gene activated human acid-β-glucocerebrosidase (velaglucerase alfa).” See also the description of the approved product and the method of manufacturing</p>

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	<p>the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 26 and the claim covers the method of manufacturing the approved product.</p>
27. The method of claim 26, wherein the cell is a fibroblast or a myoblast.	<p>As discussed above for claims 1 and 26, the method of making the approved product meets all of the limitations of the base claims.</p> <p>VPRIVTM is produced by HT-1080 cells which are fibroblasts. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 27 and the claim covers the method of manufacturing the approved product.</p>
28. The method of claim 26, wherein the cell is an immortalized cell.	<p>As discussed above for claims 1, 26 and 27, the method of making the approved product meets all of the limitations of the base claims.</p> <p>VPRIVTM is produced by an immortalized cell. See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 28 and the claim covers the method of making the</p>

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	approved product.
29. The method of claim 27, wherein the cell is an HT-1080 cell.	<p>As discussed above for claims 1, 26, 27 and 28, the method of making the approved product meets all of the limitations of the base claims.</p> <p>VPRIVTM is produced by HT-1080 cells. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 29 and the claim covers the method of manufacturing the approved product.</p>
30. The method of claim 1, wherein the cell is contacted with kifunensine in culture media.	<p>As discussed above for claim 1, the method of making the approved product meets all of the limitations of the base claim.</p> <p>VPRIVTM is produced by contacting the cells expressing hGCB with kifunensine in cell culture. See page 24, column 2 of Attachment F, "glycosylation of velaglucerase alfa is altered by using kifunensine ... during cell culture, which results in the secretion of a protein containing predominantly high-mannose type glycans." See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 30 and the claim covers the method of manufacturing the approved product.</p>

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31. The method of claim 30, wherein the hmGCB is obtained from the media in which the cell is cultured.	<p>As discussed above for claims 1 and 30, the method of making the approved product meets all of the limitations of the base claims.</p> <p>VPRIVTM is obtained from the media in which the human cell line is cultured. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 31 and the claim covers the method of manufacturing the approved product.</p>

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<p>32. A method of producing a preparation of high mannose glucocerebrosidase (hmGCB) comprising a carbohydrate chain having at least four mannose residues, the method comprising:</p> <p>providing a human cell into which a nucleic acid sequence comprising an exogenous regulatory sequence has been introduced such that the regulatory sequence is operably linked to, and regulates the expression of, an endogenous GCB coding region;</p> <p>contacting the cell with a class 1 mannosidase inhibitor such that the removal of at least one mannose residue distal to the pentasaccharide core of a precursor oligosaccharide of GCB is prevented; and allowing the cell to produce hmGCB, to thereby produce an hmGCB preparation.</p>	<p>VPRIVTM includes at least two glucocerebrosidase (GCB) proteins having at least one carbohydrate chain having four or more mannose residues. See, e.g., page 24, column 1 of Attachment F, “the predominant glycan on velaglucerase alfa is a high mannose type, with nine mannose residues”. See also Table III and Figures 7 and 8 of Attachment F. See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>VPRIVTM is produced by a human cell that includes an exogenous promoter sequence to regulate expression of an endogenous GCB coding sequence. (see, e.g., page 24, column 2 of Attachment F-“we have used gene activation in a well-characterized, continuous human cell line to produce gene activated human acid-β-glucocerebrosidase (velaglucerase alfa). Gene activation refers to targeted recombination with a promoter that activates the endogenous GlcCerase gene in the selected human cell line.” See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>VPRIVTM is produced by contacting the cells expressing hGCB with kifunensine, which is a class 1 mannosidase inhibitor, in cell culture. See page 24, column 2 of Attachment F, “glycosylation of velaglucerase alfa is altered by using kifunensine ... during cell culture, which results in the secretion of a protein</p>
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	<p>containing predominantly high-mannose type glycans". VPRIVTM includes at least 2 GCB proteins having at least one carbohydrate chain with at least one mannose residue distal to the pentasaccharide core, e.g., the GCB proteins have four or more mannose residues. See, e.g., Table III of Attachment F. See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 32 and the claim covers the method of manufacturing the approved product.</p>
33. The method of claim 32, wherein the mannosidase inhibitor prevents the removal of one or more α 1,2 mannose residue(s) distal to the pentasaccharide core.	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>The mannosidase inhibitor prevents the removal of one or more α1,2 mannose residue(s) distal to the pentasaccharide core. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 33 and the claim covers the method of manufacturing the approved product.</p>
34. The method of claim 32, wherein the mannosidase inhibitor further prevents the	As discussed above for claim 32, the method of making the approved product

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removal of one α 1,3 mannose residue distal to the pentasaccharide core.	<p>meets all of the limitations of the base claim.</p> <p>The mannosidase inhibitor further prevents the removal of one α1,3 mannose residue distal to the pentasaccharide core. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 34 and the claim covers the method of manufacturing the approved product.</p>
35. The method of claim 32, wherein the mannosidase inhibitor further prevents the removal of one α 1,6 mannose residue distal to the pentasaccharide core.	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>The mannosidase inhibitor further prevents the removal of one α1,6 mannose residue distal to the pentasaccharide core. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 35 and the claim covers the method of manufacturing the approved product.</p>
36. The method of claim 32, wherein the class 1 processing mannosidase inhibitor is kifunensine.	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p>

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	<p>VPRIVTM is produced by contacting the cells expressing hGCB with kifunensine in cell culture. See page 24, column 2 of Attachment F, "glycosylation of velaglucerase alfa is altered by using kifunensine ... during cell culture, which results in the secretion of a protein containing predominantly high-mannose type glycans". See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 36 and the claim covers the method of manufacturing the approved product.</p>
37. The method of claim 36, wherein the kifunensine is present at a concentration between about 0.05 to 20.0 µg/ml.	<p>As discussed above for claims 32 and 36, the method of making the approved product meets all of the limitations of the base claims.</p> <p>Kifunensine is present at a concentration 2.0 µg/ml in the method of making the approved product. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 37 and the claim covers the method of manufacturing the approved product.</p>

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<p>38. The method of claim 37, wherein the kifunensine is present at a concentration between about 0.1 to 2.0 µg/ml.</p>	<p>As discussed above for claims 32, 36 and 37, the method of making the approved product meets all of the limitations of the base claims.</p> <p>Kifunensine is present at a concentration 2.0 µg/ml in the method of making the approved product. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 38 and the claim covers the method of manufacturing the approved product.</p>
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<p>48. The method of claim 32, wherein the mannosidase inhibitor prevents removal of at least three mannose residues distal to the pentasaccharide core of the precursor oligosaccharide of GCB.</p>	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>Kifunensine is an inhibitor of endoplasmic reticulum mannosidase I and Golgi mannosidase I enzymes (see column 23, lines 48-51 of the '262 patent) that prevents the removal of three or more mannose residues distal to the pentasaccharide core. (see, e.g., Fig. 7 of Attachment F which shows the predominant glycan structure present on the GCB in VPRIVTM). See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p>
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	Therefore, the method of making VPRIV TM meets all of the limitations of claim 48 and the claim covers the method of manufacturing the approved product.
49. The method of claim 32, wherein the mannosidase inhibitor prevents removal of one or more mannose residues distal to the pentasaccharide core on at least two of the carbohydrate chains of hmGCB.	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>Kifunensine is an inhibitor of endoplasmic reticulum mannosidase I and Golgi mannosidase I enzymes (see column 23, lines 48-51 of the '262 patent) that prevents the removal of one or more mannose residues distal to the pentasaccharide core. (see, e.g., Fig. 7 of Attachment F which shows the predominant glycan structure present on the GCB in VPRIVTM). See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 49 and the claim covers the method of manufacturing the approved product.</p>
50. The method of claim 32, wherein at least 60% of the hmGCB of the preparation have one or more carbohydrate chains in which the removal of three or more mannose residues distal to the pentasaccharide core has been prevented.	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>At least 60% of the hmGCB of the preparation have one or more carbohydrate</p>

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	<p>chains in which the removal of three or more mannose residues distal to the pentasaccharide core has been prevented. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIV™ meets all of the limitations of claim 50 and the claim covers the method of manufacturing the approved product.</p>
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51. The method of claim 32, wherein at least 20% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>At least 20% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIV™ meets all of the limitations of claim 51 and the claim covers the method of manufacturing the approved product.</p>
52. The method of claim 51, wherein at least 40% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.	As discussed above for claims 32 and 51, the method of making the approved product meets all of the limitations of the base claims.

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	<p>At least 40% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 52 and the claim covers the method of manufacturing the approved product.</p>
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53. The method of claim 52, wherein at least 60% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.	<p>As discussed above for claims 32, 51 and 52, the method of making the approved product meets all of the limitations of the base claims.</p> <p>At least 60% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 53 and the claim covers the method of manufacturing the approved product.</p>
54. The method of claim 32, wherein at least about 80% or more of the carbohydrate chains of the hmGCB preparation have six or more mannose residues.	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>At least about 80% or more of the</p>

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	<p>carbohydrate chains of the hmGCB preparation have six or more mannose residues. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 54 and the claim covers the method of manufacturing the approved product.</p>
57. The method of claim 32, wherein the cell is a fibroblast or a myoblast.	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>VPRIVTM is produced by an HT-1080 cell line, which is a fibroblast. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 57 and the claim covers the method of manufacturing the approved product.</p>
58. The method of claim 32, wherein the cell is an immortalized cell.	<p>As discussed above for claim 32, the method of making the approved product meets all of the limitations of the base claim.</p> <p>VPRIVTM is produced by an immortalized cell. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p>

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	Therefore, the method of making VPRIV TM meets all of the limitations of claim 58 and the claim covers the method of making the approved product.
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59. The method of claim 58, wherein the cell is an HT-1080 cell.	<p>As discussed above for claims 32 and 58, the method of making the approved product meets all of the limitations of the base claims.</p> <p>VPRIVTM is produced by an HT-1080 cell line. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 59 and the claim covers the method of manufacturing the approved product.</p>
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<p>60. The method of claim 36, wherein the cell is contacted with kifunensine in culture media.</p>	<p>As discussed above for claims 32 and 36, the method of making the approved product meets all of the limitations of the base claims.</p> <p>VPRIVTM is produced by contacting the cells expressing hGCB with kifunensine in cell culture. See page 24, column 2 of Attachment F, "glycosylation of velaglucerase alfa is altered by using kifunensine ... during cell culture, which results in the secretion of a protein containing predominantly high-mannose type glycans" See also the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 60 and the claim covers the method of manufacturing the approved product.</p>
<p>61. The method of claim 60, wherein the hmGCB is obtained from the media in which the cell is cultured.</p>	<p>As discussed above for claims 32, 36, and 60, the method of making the approved product meets all of the limitations of the base claims.</p> <p>VPRIVTM is obtained from the media that the cell line is cultured. See the description of the approved product and the method of manufacturing the same in this section (9)(iii).</p> <p>Therefore, the method of making VPRIVTM meets all of the limitations of claim 61 and the claim covers the method of</p>

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	manufacturing the approved product.
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**(10) Relevant Dates Under 35 U.S.C. § 156 for Determination of
Applicable Regulatory Review Period [1.740(a)(10)]**

The relevant dates and information pursuant to 35 U.S.C. § 156(g) to enable the Secretary of Health and Human Services to determine the applicable regulatory review period are as follows:

Patent Issue Date:

U.S. Patent No. 7,138,262 B1 issued on November 21, 2006.

***(i)(A) IND Effective Date and IND number [35 U.S.C. §156(g)(1)(B)(i); 37
C.F.R. §1.740(a)(10)(i)(A)]***

The effective date of IND 61,220 was May 20, 2004.

An IND was by submitted by Transkaryotic Therapies, Inc. to FDA and received by FDA on December 31, 2003. It was assigned number IND 61,220. A copy of the letter from FDA to Transkaryotic Therapies, Inc., dated January 12, 2004, providing the IND number and showing the date of receipt by FDA of the IND is provided in Attachment G. On January 28, 2004, FDA notified Transkaryotic Therapies, Inc. that a modification to the protocol was necessary. A written record of the discussion is provided in Attachment G1. Transkaryotic Therapies, Inc submitted an amendment to the protocol on March 11, 2004, see letter from Transkaryotic Therapies, Inc. to FDA, dated March 11, 2004, concerning amendment of protocol, provided in Attachment G2. On May 20, 2004, FDA notified Transkaryotic Therapies, Inc. that it could proceed, see FDA communication to Transkaryotic Therapies, Inc. dated May 20, 2004, concerning amendment to protocol, a copy of which is provided in Attachment G3.

On November 20, 2006, FDA notified Shire Human Genetics Therapies, Inc. that IND 61,220 was on clinical hold. A copy of the letter from the FDA to Shire Human Genetics Therapies, Inc. showing the date the FDA notified Shire Human Genetics

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Therapies, Inc. of the clinical hold is provided as Attachment H. The FDA removed the clinical hold on December 21, 2006. A copy of the letter dated December 21, 2006 from the FDA to Shire Human Genetics Therapies, Inc. indicating that the hold had been removed and the protocol could be initiated is provided as Attachment I.

Thus, as set out above, the date that an exemption under §505(i) of the Federal Food, Drug and Cosmetic Act became effective (i.e., the date that an investigational new drug application (IND) became effective for VPRIVTM) was May 20, 2004.

(i)(B) NDA Submission Date [35 U.S.C. §156(g)(1)(B)(i); 37 C.F.R.

§1.740(a)(10)(i)(B)] The NDA was submitted on a rolling basis. The initial portion of the NDA was submitted by Shire Human Genetic Therapies, Inc. to the FDA on July 30, 2009. The final portion was submitted on August 31, 2009. This date is used in the calculations provided herein. The NDA was assigned number NDA 22575. A copy of a letter from FDA to Shire Human Genetic Therapies, Inc., dated September 14, 2009, acknowledging receipt of the final submission of the NDA application is provided as Attachment J.

(i)(C) NDA Approval Date [35 U.S.C. §156(g)(1)(B)(ii); 37 C.F.R.

§1.740(a)(10)(i)(C)]

The FDA approved NDA 22575 authorizing the marketing of VPRIVTM on February 26, 2010. VPRIVTM was approved under the Department of Health and Human Services (DHHS). A copy of the approval letter from FDA to Shire Human Genetic Therapies, Inc., dated February 26, 2009 is provided as Attachment C.

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MANNOSE PROTEINS**(11) Summary of Significant Events During Regulatory Review Period****[1.740(a)(11)]**

A brief description of the significant activities undertaken by the marketing applicant during the applicable regulatory review period with respect to VPRIVTM and the dates applicable to these significant activities are set forth in a chronology of events provided below.

Date	IND or NDA Serial No. (if applicable)	Other Contact	Description
06 April 2001			Pre-IND Teleconference Request
18 Nov. 2003			Pre-IND Meeting
30 Dec. 2003	IND 61,220 Serial 000		Submission of Original IND (including general information on Gaucher disease, nonclinical data, manufacturing info., and Phase I/II study - TKT025 New Protocol and IB).
19 Jan. 2004		FDA letter	FDA Correspondence: Acknowledgement of Receipt of IND and assignment of IND number
28 Jan 2004		TCR Contact Report	FDA Medical Officer request protocol amendment as discussed at the Pre-IND meeting.
28 Jan 2004	IND 61,220 Serial 001		Response to email dated 13 Jan. 2004 containing questions on clarification of age, inclusion criteria, and genotyping.
11 Mar. 2004	IND 61,220 Serial 002		Protocol Amendment: Amendment 2 of Clinical Protocol TKT025
07 April 2004	IND 61,220 Serial 003		Protocol Amendment: New Investigator for TKT025 and Blinding procedures used in TKT025 .
20 May 2004		TCR Contact Report	FDA Medical Officer says it's safe to proceed with the blinding procedure as amended in Serial 003 dated 07 April 2004 for Study TKT025 .
25 Aug. 2004		FDA letter	Re: Completion of IND Preclinical Pharm/Tox review and comments/recommendations
02 Nov. 2004	IND 61,220 Serial 007		Protocol Amendment: TKT025 Protocol Amendment 4 and New Protocol TKT025EXT
24 Nov. 2004	IND 61,220 Serial 008		Information Amendment: Comparability Protocol – comprehensive plan for evaluating changes to manufacturing process for drug substance (switch from 3x 30L Bioreactor to 100 L Bioreactor).
06 Apr.	IND 61,220 Serial		Information Amendment: Pharm/Tox: Final Study

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Date	IND or NDA Serial No. (if applicable)	Other Contact	Description
2005	017		Reports: Rat & Monkey 6-Month tox studies.
04 Nov. 2005	IND 61,220 Serial 023		Type B Meeting (End of Phase II) Request
12 Dec. 2005	IND 61,220 Serial 024		End of Phase II Briefing Document
09 Jan. 2006		FDA Fax	FDA responses to EOP II Meeting questions
11 Jan. 2006			End of Phase II Meeting
07 Feb. 2006		FDA letter	Official FDA Minutes from EOPII meeting 11 Jan. 2006
30 Mar. 2006	IND 61,220 Serial 029		Information Amendment: CMC: Manufacturing process modifications: switch from 3x 30L to 500 L scale up (AF1 process).
12 April 2006	IND 61,220 Serial 030		Information Amendment: Pharm/Tox: supportive information to justify Nonclinical Development Program and request for teleconference
02 May 2006	IND 61,220 Serial 031		Type A Meeting Request to discuss adequacy of nonclinical development program to initiate P3 studies and support a NDA.
18 May 2006	IND 61,220 Serial 032		Type C Meeting on 16 June 2006: Pharm/Tox Briefing Package
15 June 2006		FDA Fax	FDA Correspondence: 16 June 2006 teleconference not necessary, based on FDA'S initial responses to questions.
23 June 2006		FDA letter	FDA Correspondence: Comments and request for additional information, re: amendment dated 12 April 2006, IND Serial 030.
12 July 2006		FDA letter	FDA Correspondence: Acknowledgement of Shire's decision to accept FDA's written responses in lieu of meeting.
28 July 2006	IND 61,220 Serial 034		Information Amendment: Pharm/Tox: Responses to FDA comments and requests to 23 June fax, rat and rabbit studies.
03 Aug. 2006	IND 61,220 Serial 035		Information Amendment: CMC: Description and comparability data of AF1 process material
22 Sept. 2006	IND 61,220 Serial 037		Protocol Amendment: New Protocol, Phase 2/3 Clinical Protocol TKT032
16 Nov. 2006	IND 61,220 Serial 039		Protocol Amendment: New Protocol, Phase 3 Clinical Protocol TKT034
20 Nov. 2006		TCR Contact Report	Teleconference: IND put on clinical hold over concerns about product comparability.
28 Nov.		FDA Fax	FDA Correspondence: Full Clinical Hold Letter

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Date	IND or NDA Serial No. (if applicable)	Other Contact	Description
2006			
28 Nov. 2006	IND 61,220 Serial 040		Type A Meeting Request, response to Clinical Hold.
29 Nov. 2006		TCR Contact Report	Teleconference: response to FDA concerns relating to DS comparability resulting in Full Clinical Hold.
30 Nov. 2006	IND 61,220 Serial 041		Complete Response to Full Clinical Hold Letter relating to DS comparability.
30 Nov. 2006	IND 61,220 Serial 042		Request for Partial Waiver of the Full Clinical Hold.
01 Dec. 2006		TCR Contact Report	Teleconference: FDA agrees to move the Full Clinical Hold to a partial hold after reviewed Shire's response.
07 Dec. 2006		FDA letter	FDA Correspondence: Partial Clinical Hold Letter
12 Dec. 2006	IND 61,220 Serial 043		Response to Clinical/Statistical non-hold issues raised in full Clinical Hold Letter (Study TKT032)
14 Dec. 2006	IND 61,220 Serial 044		Protocol Amendment for Study TKT025EXT.
21 Dec. 2006		FDA letter	FDA Correspondence: Removal of Partial Clinical Hold Note: all clinical issues have been resolved.
26 Feb. 2007	IND 61,220 Serial 047		Protocol Amendment: New Protocol HGT-GCB-039 and New Investigator for TKT032
04 Sept. 2007		FDA letter	FDA Correspondence: Request for Information-Study TKT034
24 Sept. 2007	IND 61,220 Serial 055		Response to FDA request for Information-Study TKT032
10 Dec. 2007	IND 61,220 Serial 057		Response to FDA Request for Information-Study TKT034
11 Dec. 2007	IND 61,220 Serial 058		Information Amendment: Comparability Protocol for drug substance cell culture scale-up (AF2) vs. AF1 process
19 Dec 2007	IND 61,220 Serial 060		Protocol Amendment: New Protocol HGT-GCB-044 (Extension study for TKT032, TKT034 and HGT- GCB-039).
03 Sept. 2008	IND 61,220 Serial 070		Information Amendment: Description and comparability data of AF2 process material
06 Oct. 2008	IND 61,220 Serial 072		Information Amendment: Description and comparability data of 200 U/vial presentation
30 April 2009			Request for Orphan Drug Designation to FDA OOPD
08 June 2009	IND 61,220 Serial 081		Type B Meeting Request: Pre-NDA Meeting
08 June 2009		FDA OOPD	FDA OOPD Correspondence: Orphan Drug Designation Granted, US ODD #09-2835

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Date	IND or NDA Serial No. (if applicable)	Other Contact	Description
		letter	
30 June 2009	IND 61,220 Serial 082		Submission of Treatment Protocol HGT-GCB-058
30 June 2009	IND 61,220 Serial 083		Request for Fast Track Designation
01 July 2009		FDA letter	FDA Correspondence: Type B Pre-NDA meeting Granted on 10 August, 2009
08 July 2009		FDA letter	FDA Correspondence: Acknowledgement of Fast Track designation Request
15 July 2009		FDA letter	FDA Correspondence: Fast Track Designation Granted
23 July 2009		TCR Contact Report	Plans for rolling NDA for velaglucerase alfa
27 July 2009	IND 61,220 Serial 088		Request for Submission of Portions of an NDA Application
29 July 2009		WHO Collaboratin g Centre for Drug Statistics Methodology	ATC application for velaglucerase alfa (Ref: 09/1527- 2/EPLI/TUGR). ATC Proposed Code: A16AB10 velaglucerase alfa.
30 July 2009		FDA letter	FDA Correspondence: Acknowledgement to Proceed with Treatment Protocol HGT-GCB-058
30 July 2009	NDA 022575, Sequence 0000		Submission of 1st wave Rolling NDA, including M3 (complete), M4 (complete), M5 (partial). And request for Priority Review of NDA
08 Aug. 2009		FDA Fax	FDA Correspondence: FDA preliminary response for Pre- NDA Meeting Briefing Package
10 Aug. 2009			Pre-NDA Meeting
31 Aug. 2009	NDA 022575, Sequence 0001		Submission of 2nd wave of Rolling NDA, including M1, M2 (complete), M3 (update), M5 (complete).
14 Sept. 2009		FDA email, fax, letter	FDA Correspondence: FDA Acknowledgement Letter of NDA Submission
22 Sept. 2009	NDA 022575, Sequence 0003		Request for Proprietary Name Review
01 Oct. 2009	NDA 022575, Sequence 0005		Trade Name Request –Labeling Supplement
30 Oct. 2009		FDA letter	FDA Correspondence: Filing Communication – Priority Review Granted, and a list of review questions included.
03 Nov. 2009	NDA 022575, Sequence 0011		Location of data to support Orphan Drug Designation
19 Nov.		FDA Fax	Request Clinical Information

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Date	IND or NDA Serial No. (if applicable)	Other Contact	Description
2009			
20 Nov. 2009		FDA Email String	FDA Inspection Announcement Letters - 2 sites Inspections (Dec 6-10, 14-18, 2009)
20 Nov. 2009	NDA 022575, Sequence 0012		Partial Response to Request for Information: FDA Questions dated 30 Oct. 2009
01 Dec. 2009	NDA 022575, Sequence 0013		3 Month Safety Update
04 Dec. 2009		Email String	FDA BIMO Visit to Shire HGT LTP Site (Dec 08, 2009)
04 Dec. 2009	NDA 022575, Sequence 0014		Complete Response to Request for Information: FDA Questions dated 30 Oct. 2009 and 19 Nov. 2009
11 Dec. 2009		FDA Fax	Request CMC, Clinical Information
16 Dec. 2009		FDA letter	Proprietary name request : Conditional acceptance
18 Dec. 2009	NDA 022575, Sequence 0016		Response to Request for Information: FDA letter dated 11 Dec. 2009 (CMC)
22 Dec. 2009	NDA 022575, Sequence 0017		Stability update (drug substance and drug product)
31 Dec. 2009	NDA 022575, Sequence 0018		Response to Request for Information: Quality and Efficacy
13 Jan. 2010	NDA 022575, Sequence 0019		Response to Request for Information: CMC Questions of 23 Dec. 2009 and 07 Jan 2010 letters
14 Jan. 2010	NDA 022575, Sequence 0020		Response to to Request for Information: Clinical Questions of 23 Dec. 2009 Fax
15 Jan. 2010	NDA 022575, Sequence 0021		Response to telephone request of 15 Jan. 2010 – CMC information
26 Jan. 2010	NDA 022575, Sequence 0022		Response to Request for Information regarding inspections: Responses to FDA Form 483 in Paraguay, Israel, Shire HGT (300 PW)
27 Jan. 2010	NDA 022575, Sequence 0023		Response to 22 Jan. 2010 FDA Request for CMC Information
29 Jan. 2010		FDA email & letter	FDA comments on US PI
01 Feb. 2010	NDA 022575, Sequence 0024		Response to 27 Jan. 2010 FDA Request for Clinical Information
08 Feb. 2010	NDA 022575, Sequence 0025		Response to 03 Feb. 2010 FDA Fax Request for Clinical Information
09 Feb. 2010	NDA 022575, Sequence 0026		Response to FDA labeling question dated 29 Jan. 2010
10 Feb. 2010		FDA email &	FDA comments on labeling-carton labeling and container labels

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Date	IND or NDA Serial No. (if applicable)	Other Contact	Description
		letter	
17 Feb. 2010		FDA email	Response to FDA fax 10 Feb 2010 on labeling-carton label and vial labels
17 Feb. 2010	NDA 022575, Sequence 0027		Response to FDA labeling question dated 10 Feb. 2010 (carton and container comments)
18 Feb. 2010		FDA Fax	FDA comments on labeling-carton and container labels
19 Feb. 2010	NDA 022575, Sequence 0028		Response to FDA labeling comments 17 Feb 2010 and carton and container label comments 18 Feb 2010
25 Feb. 2010	NDA 022575, Sequence 0029		Information Amendment: Final Post-Marketing Commitments and Final Labeling Text (Company agreed PMCs and labeling text)
25 Feb. 2010	NDA 022575, Sequence 0030		Information Amendment: Post-Marketing Commitments and Draft Labeling Text
26 Feb. 2010		FDA Action Letter	NDA Approval Letter

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(12) Statement Concerning Eligibility for and Duration of Extension

Sought Under 35 U.S.C. § 156 [37 C.F.R. §1.740(a)(12)]

(i) Applicant is of the opinion that U.S. Patent No. 7,138,262 B1 is eligible for extension of the patent term under 35 U.S.C. § 156 of 687 days and should be extended until July 6, 2022. It satisfies all requirements for such extension including:

(a) 35 U.S.C. § 156(a) - U.S. Patent No. 7,138,262 B1 claims a method of manufacturing the approved product, VPRIV™.

(b) 35 U.S.C. § 156(a)(1) - U.S. Patent No. 7,138,262 B1 has not expired before submission of this application.

(c) 35 U.S.C. § 156(a)(2) - The term of U.S. Patent No. 7,138,262 B1 has never been extended under 35 U.S.C. § 156(e)(1).

(d) 35 U.S.C. § 156(a)(3) - The application for patent term extension is submitted by the owner of record of the patent in accordance with the requirements of paragraphs (1) through (4) of 35 U.S.C. § 156(d) and the rules of the Patent and Trademark Office.

(e) 35 U.S.C. § 156(a)(4) - The product VPRIV™ has been subject to a regulatory review period before its commercial marketing or use.

(f) 35 U.S.C. § 156(a)(5)(A) - The commercial marketing or use of the product VPRIV™ after the regulatory review period is the first permitted commercial marketing or use under the provisions of § 505(b) of the Federal Food, Drug, and Cosmetic Act under which such regulatory review period occurred.

(g) 35 U.S.C. § 156(c)(4) - No other patent has been extended for the same regulatory review period for the product VPRIV™.

(h) This application is being submitted within 60 days of regulatory agency approval.

(i) This application otherwise complies with all requirements of 35 U.S.C. § 156 and all applicable rules and procedures.

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(12)(ii) Applicant respectfully submits that the length of the extension of patent term for U.S. Patent No. 7,138,262 B1 is 687 days pursuant to 35 U.S.C. § 156(c).

The length of the extension was determined pursuant to 37 C.F.R. § 1.775 as follows (the remainder of this section (12)(ii) is numbered so as to correspond to the numbering in 37 C.F.R. § 1.775):

(c) The regulatory review period under 35 U.S.C. § 156(g)(1)(B) is a total of 2,110 days, which is the sum of (1) and (2) below:

(1) The period of review under 35 U.S.C. § 156(g)(1)(B)(i), which is the number of days in the period beginning on the date the exemption became effective (May 20, 2004) and ending on the date an application was initially submitted (August 31, 2009), which is 1,930 days; and

(2) The period of review under 35 U.S.C. § 156(g)(1)(B)(ii), which is the number of days in the period beginning on the date the application was initially submitted (August 31, 2009) and ending on the date such application was approved (February 26, 2010), which is 180 days.

(d) The term of the patent as extended for a human drug, antibiotic drug or human biological product is determined by:

(1) Subtracting from the number of days determined to be in the regulatory review period, which is 2,110:

(i) The number of days in the regulatory review period which were on or before the date on which the patent issued (November 21, 2006) which is 916 days; and

(ii) The number of days in the period of (c)(1) and (c)(2) above during which applicant did not act with due diligence, which is zero (0) days; and

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(iii) One-half the number of days determined in subparagraph (c)(1) above after that period is reduced by subparagraph (d)(1)(i) and (d)(1)(ii) which, is $(1,930-916-0)/2$, or 507 days.

Thus, the number of days determined in subparagraph (c) above (2,110) is reduced by 1,423 $(916+507)$ days, for a total of 687 days;

(2) Adding the number of days as determined in subparagraph (d)(1), (687 days), to the original term of the patent (August 18, 2020) which results in the date of July 6, 2022.

(3) By adding fourteen (14) years to the date of approval of the New Drug Application (NDA 22575) which results in the date of February 26, 2024;

(4) By comparing the dates for the ends of the periods obtained pursuant to paragraphs (d)(2) and (d)(3) and selecting the earlier, which is July 6, 2022;

(5) (i) Since U.S. Patent No. 7,138,262 B1 issued after September 24, 1984, by adding 5 years to the original expiration date of the patent or any earlier date set by terminal disclaimer, which is August 18, 2025; and (ii) By comparing the dates obtained pursuant to paragraphs (d)(4) and (d)(5)(i) of this section with each other and selecting the earlier date, which is July 6, 2022.

Thus, the patent is entitled to extension until July 6, 2022.

(13) Statement Pursuant to 37 C.F.R. § 1.740(a)(13)

Applicant acknowledges a duty to disclose to the Director of the United States Patent and Trademark Office and the Secretary of Health and Human Services any information which is material to the determination of entitlement to the extension sought, e.g., as that duty is defined in 37 C.F.R. § 1.765.

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(14) Applicable Fee [1.740(a)(14)]

The prescribed fee for receiving and acting upon this application is attached as a check in the amount of \$1,120.00. The Director is authorized to charge any additional fees required by this application to Deposit Account No. 50/2762, referencing attorney docket number S2071-701019.


(15) Name and Address for Correspondence [1.740(a)(15)]

All correspondence and inquiries may be directed to the undersigned, whose address, telephone number and fax number are as follows:

Laurie Butler Lawrence
Lando & Anastasi, LLP
One Main Street
Cambridge, MA 02142
Phone: 617-395-7000
Fax: 617-395-7070

Enclosed is a certification that the application for extension of patent term under 35 U.S.C. § 156 including its attachments and supporting papers is being submitted as one original and two (2) copies thereof (Attachment K) in compliance with 37 C.F.R. § 1.740(b).

Respectfully submitted,

By: 
Laurie Butler Lawrence, Reg. No. 46,593
LANDO & ANASTASI, LLP
One Main Street
Cambridge, Massachusetts 02142
United States of America
Telephone: 617-395-7000
Facsimile: 617-395-7070

In re U.S. Patent No.: 7,138,262 B1
Issued: November 21, 2006
Inventors: Peter Francis Daniel
Assignee: Shire Human Genetic Therapies,
Inc.

Attorney Docket No.: S2071-701019/0013US

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Date: April 22, 2010

Attachments:

Power of Attorney (Attachment A)

Package Insert for VPRIV™ (Attachment B)

NDA approval letter from FDA to Shire Human Genetic Therapies, Inc., dated
February 26, 2010 (with enclosure) (Attachment C)

U.S. Patent No. 7,138,262 B1 (Attachment D)

Maintenance Fee Statement (Attachment E)

Brumshtein et al. (2010) Glycobiology 20(1):24-32 (Attachment F)

Letter from FDA to Transkaryotic Therapies, Inc., dated January 12, 2004,
providing the IND number and showing the date of receipt by FDA of the IND
(Attachment G)

A written record of the discussion that occurred on January 28, 2004 regarding
modification of the protocol (Attachment G1)

Letter from Transkaryotic Therapies, Inc. to FDA dated March 11, 2004,
concerning amendment of protocol (Attachment G2)

FDA communication to Transkaryotic Therapies, Inc., dated May 20, 2004,
concerning amendment to protocol (Appendix G3).

Letter from FDA to Shire Human Genetics Therapies, Inc. indicating the date the
IND was put on clinical hold (Attachment H)

Letter from FDA to Shire Human Genetics Therapies, Inc., dated December 21,
2006, removing the clinical hold and indicating that the protocol can be initiated
(Attachment I)

Letter from FDA to Shire Human Genetics Therapies, Inc., dated September 14,
2009, acknowledging receipt of the final submission of the NDA (Attachment J)

Certification of Copies of Application Papers (Attachment K)

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment A

Power of Attorney

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

**REVOCATION OF PRIOR POWERS OF ATTORNEY
and
NEW POWER OF ATTORNEY**

Sir:

The undersigned, Shire Human Genetic Therapies, Inc., a Delaware Corporation, assignee of the entire right, title and interest for all of the patents and patent applications identified in the attached Schedule A, hereby revokes all previous powers of attorney or authorizations of agent given in the identified patents and patent applications and in any divisional, continuing, substitute, renewal, reexamination, or reissue applications thereof, and appoints all practitioners of Lowrie, Lando & Anastasi, LLP associated with Customer Number:

37462

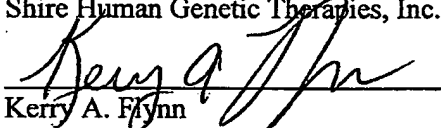
as assignee's attorneys or agents with full power of substitution to take any and all action necessary with regard to the identified patents and patent applications, and with regard to any divisional, continuing, substitute, renewal or reissue applications thereof.

Please address all telephone calls to Laurie Butler Lawrence at telephone no. (617) 395-7000.

Please forward all correspondence to the correspondence address associated with

Customer Number: **37462**

Shire Human Genetic Therapies, Inc.


By: 
Name: Kerry A. Flynn
Title: Vice President, Intellectual Property

Dated: April 14, 2008

ASSIGNEE CERTIFICATION

Attached to this power is a Certificate Under 37 CFR 3.73(b).

Dated: April 17, 2008


Natalie A. Lissy, Reg. No. 59,651
LOWRIE, LANDO & ANASTASI, LLP
Riverfront Office Park
One Main Street
Cambridge, MA 02142
(617) 395-7000

SCHEDULE A

U.S. Patents:

<u>U.S. PATENT NO.</u>	<u>ISSUE DATE</u>	<u>ATTORNEY'S DOCKET NO.</u>
6,924,365	08/02/2005	S2071-700410
7,229,793	06/12/2007	S2071-700719
6,569,681	05/27/2003	S2071-700919
7,138,262	11/21/2006	S2071-701019
5,965,125	10/12/1999	S2071-701419
6,472,181	10/29/2002	S2071-701440
6,582,391	06/24/2003	S2071-701441
6,083,725	07/04/2000	S2071-701510
6,566,099	05/20/2003	S2071-701520
7,122,354	10/17/2006	S2071-701521
6,395,884	05/28/2002	S2071-701540
5,817,789	10/06/1998	S2071-701619
6,027,921	02/22/2000	S2071-701640
6,262,026	07/17/2001	S2071-701641
6,858,578	02/22/2005	S2071-701642
6,419,920	07/16/2002	S2071-701730
6,458,574	10/01/2002	S2071-702030

SCHEDULE A

U.S. Patent Applications:

<u>U.S. APPLICATION NO.</u>	<u>FILING DATE</u>	<u>ATTORNEY'S DOCKET NO.</u>
11/581,979	10/17/2006	S2071-701040
11/028,850	01/03/2005	S2071-701620
10/160,452	05/31/2002	S2071-701740
10/165,060	07/07/2002	S2071-702040
11/403,618	04/13/2006	S2071-702540
11/671,588	02/06/2007	S2071-702719
10/775,678	02/10/2004	S2071-702810
08/712,614	09/13/1996	S2071-703119
10/423,225	04/25/2003	S2071-702510
09/686,497	10/11/2000	S2071-701319
11/924,804	10/26/2007	S2071-701320
11/925,125	10/26/2007	S2071-701321
11/925,167	10/26/2007	S2071-701322
11/928,247	10/30/2007	S2071-701323
10/165,968	06/10/2002	S2071-702020
60/375,584	04/25/2002	S2071-702500
60/771,555	02/07/2006	S2071-702700
10/968,870	10/18/2004	S2071-701020

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

STATEMENT UNDER 37 CFR 3.73(b)Applicant/Patent Owner: Peter Francis Daniel et al.Application No./Patent No.: 7,138,262 Filed/Issue Date: 11/21/2006Entitled: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH MANNOSE PROTEINSShire Human Genetics Therapies, Inc., a Delaware Corporation
(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that it is:

1. ☒ the assignee of the entire right, title, and interest; or
2. ☐ an assignee of less than the entire right, title and interest
(The extent (by percentage) of its ownership interest is _____ %)

in the patent application/patent identified above by virtue of either:

A. ☐ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel _____, Frame _____, or for which a copy thereof is attached.

OR

B. ☒ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: Peter Francis Daniel et al. To: Transkaryotic Therapies, Inc.
The document was recorded in the United States Patent and Trademark Office at
Reel 011662, Frame 0815, or for which a copy thereof is attached.
2. From: Transkaryotic Therapies, Inc. To: Shire Human Genetics Therapies, Inc.
The document was recorded in the United States Patent and Trademark Office at
Reel 018224, Frame 0390, or for which a copy thereof is attached.
3. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at
Reel _____, Frame _____, or for which a copy thereof is attached.

☐ Additional documents in the chain of title are listed on a supplemental sheet.

☒ As required by 37 CFR 3.73(b)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (i.e., a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

<u>/Natalie A. Lissy/</u>	<u>April 24, 2008</u>
Signature	Date
<u>Natalie A. Lissy, Reg. No. 59,651</u>	<u>617-395-7000</u>
Printed or Typed Name	Telephone Number
<u>Attorney</u>	
Title	

This collection of information is required by 37 CFR 3.73(b). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

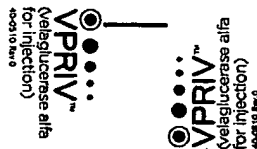
Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment B

Package Insert for VPRIVTM



HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use VPRIV safely and effectively. See full prescribing information for VPRIV.

VPRIV (velaglucease alfa for injection)
Initial U.S. Approval: 2010

INDICATIONS AND USAGE

VPRIV (velaglucease alfa for injection) is a hydrolytic lysosomal glucosaminidase-specific enzyme indicated for long-term enzyme replacement therapy (ERT) for pediatric and adult patients with type 1 Gaucher disease (1).

DOSE AND ADMINISTRATION

- 60 Units/kg administered every other week as a 60-minute intravenous infusion (2).
- Patients currently being treated with imiglucerase for Gaucher disease can be switched to VPRIV. Patients previously treated on a stable dose of imiglucerase are recommended to begin treatment with VPRIV at that same dose when they switch from imiglucerase to VPRIV (2).
- Physicians can make dosage adjustments based on achievement and maintenance of each patient's therapeutic goals. Clinical trials have evaluated doses ranging from 15 Units/kg to 60 Units/kg every other week (2).

DOSE FORMS AND STRENGTHS

- Lyophilized powder to be reconstituted and diluted for infusion (3).

- Available in 200 Units and 400 Units single-use vials (3).

CONTRAINDICATIONS

- None (4).

WARNINGS AND PRECAUTIONS

- Hypersensitivity reactions: Treatment with VPRIV should be carefully re-evaluated in the presence of significant evidence of hypersensitivity to the product (5.1).
- Infusion-related reactions (5.2).

ADVERSE REACTIONS

- The most common adverse reactions during clinical studies were infusion-related reactions (5.2, 6.1).
- Other commonly observed adverse reactions in a 10% of patients were headache, dizziness, abdominal pain, nausea, back pain, joint pain, upper respiratory tract infection, activated PTI, pyrexia, and pyruvate (6.1).

To report SUSPECTED ADVERSE REACTIONS, contact Shire Human Genetic Therapies, Inc. at the OnePathSM phone 1-866-888-0660 or MedInfo@shire.com, or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch. See Section 17 for PATIENT COUNSELING INFORMATION.

Revised: 02/2010

FULL PRESCRIBING INFORMATION: CONTENTS

- 1 INDICATIONS AND USAGE
- 2 DOSAGE AND ADMINISTRATION
 - 2.1 Recommended Dose
 - 2.2 Preparation and Administration Instructions
- 3 DOSAGE FORMS AND STRENGTHS
- 4 CONTRAINDICATIONS
- 5 WARNINGS AND PRECAUTIONS
 - 5.1 Hypersensitivity Reactions
 - 5.2 Infusion-related Reactions
- 6 ADVERSE REACTIONS
 - 6.1 Clinical Studies Experience
- 7 DRUG INTERACTIONS
- 8 USE IN SPECIFIC POPULATIONS
 - 8.1 Pregnancy - Category B
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- 15 REFERENCES
- 16 HOW SUPPLIED/STORAGE AND HANDLING
 - 16.1 Storage
- 17 PATIENT COUNSELING INFORMATION

*Sections or subsections omitted from the full prescribing information are not listed.

VPRIVTM (velaglucease alfa for injection)

FULL PRESCRIBING INFORMATION

1 INDICATIONS AND USAGE

VPRIV (velaglucease alfa for injection) is a hydrolytic lysosomal glucosaminidase-specific enzyme indicated for long-term enzyme replacement therapy (ERT) for pediatric and adult patients with type 1 Gaucher disease.

2 DOSAGE AND ADMINISTRATION

2.1 Recommended Dose

The recommended dose is 60 Units/kg administered every other week as a 60-minute intravenous infusion. Patients currently being treated with imiglucerase for type 1 Gaucher disease may be switched to VPRIV. Patients previously treated on a stable dose of imiglucerase are recommended to begin treatment with VPRIV at that same dose when they switch from imiglucerase to VPRIV. Dosage adjustments can be made based on achievement and maintenance of each patient's therapeutic goals. Clinical studies have evaluated doses ranging from 15 Units/kg to 60 Units/kg every other week. VPRIV should be administered under the supervision of a healthcare professional.

2.2 Preparation and Administration Instructions

Use aseptic technique

VPRIV is a lyophilized powder, which requires reconstitution and dilution, and is intended for intravenous infusion only. VPRIV contains no preservatives and stable single-use vials. Once an vial is opened, VPRIV should be prepared as follows:

Determine the number of vials to be reconstituted based on the individual patient's weight and the prescribed dose. Follow the instructions in Table 1 for reconstitution.

Table 1: Reconstitution Instructions

	200 Units/vial	400 Units/vial
Volumes of Sterile Water for Injection, USP, for reconstitution	2.2 mL	4.3 mL
Concentration after reconstitution	100 Units/mL	100 Units/mL
Withdrawal volume	2 mL	4 mL

Upon reconstitution, mix well gently. DO NOT SHAKE. Prior to further dilution, visually inspect the solution in the vial; the solution should be clear to slightly opalescent and colorless. Do not use if the solution is discolored or if foreign particulate matter is present. Withdraw the calculated volume of drug from the appropriate number of vials and dilute the total volume required in 100 mL of 0.9% sodium chloride solution suitable for IV administration. Mix gently. DO NOT SHAKE.

VPRIV should be administered over 60 minutes. VPRIV must not be infused with other products in the same infusion tubing as the compatibility of solutions with other products has not been evaluated. The diluted solution should be filtered through an in-line low protein-binding 0.2 µm filter during administration.

As VPRIV contains no preservatives, once reconstituted the product should be used immediately. If immediate use is not possible, the reconstituted or diluted product may be stored for up to 24 hours at 2 to 8°C (36 to 46°F). Do not freeze. Protect from light. The infusion should be completed within 24 hours of reconstitution of vials.

3 DOSAGE FORMS AND STRENGTHS

VPRIV is a sterile, white to off-white, lyophilized powder for reconstitution with Sterile Water for Injection, USP, to yield a final concentration of 100 Units/mL.

VPRIV is available in 200 Units and 400 Units single-use vials.

4 CONTRAINDICATIONS

None.

5 WARNINGS AND PRECAUTIONS

5.1 Hypersensitivity Reactions

Hypersensitivity reactions have been reported in patients in clinical studies with VPRIV [see Adverse Reactions (6.1)]. As with any recombinant protein product, hypersensitivity reactions are possible. Therefore appropriate medical support should be readily available when VPRIV is administered. If a severe reaction occurs, current medical standards for emergency treatment are to be followed.

Treatment with VPRIV should be approached with caution in patients who have exhibited symptoms of hypersensitivity to the active ingredient or excipients in the drug product or to other enzyme replacement therapy.

5.2 Infusion-related Reactions

Infusion-related reactions were the most commonly observed adverse reactions in patients treated with VPRIV in clinical studies. The most commonly observed symptoms of infusion-related reactions were headache, dizziness, hypotension, hypertension, nausea, back-pain, and pyrexia. Generally the infusion-related reactions were mild and in intravenous patients, onset occurred mainly during the first 6 months of treatment and tended to occur less frequently with time.

The management of infusion-related reactions should be based on the severity of the reaction, e.g. slowing the infusion rate, treatment with antihistamines such as antihistamines, antipyretics and/or corticosteroids, and/or stopping and resuming treatment with increased infusion time. Treatment with antihistamines and/or corticosteroids may prevent subsequent reactions in those cases where symptomatic treatment was required. Patients were not routinely pre-medicated prior to infusion of VPRIV during clinical studies.

6 ADVERSE REACTIONS

6.1 Clinical Studies Experience

The data described below reflect exposure of 84 patients with type 1 Gaucher disease who received VPRIV at doses ranging from 15 Units/kg to 60 Units/kg every other week in 3 clinical studies. Fifty-four (64%) patients were naive to ERT and received VPRIV for 8 months and 40 patients switched from imiglucerase to VPRIV treatment and received VPRIV for 12 months [see Clinical Studies (14)]. Patients were between 4 and 71 years old at time of first treatment with VPRIV, and included 45 male and 39 female patients.

The most common adverse reactions in patients treated with VPRIV were hypersensitivity reactions [see Warnings and Precautions (5.1)].

The most commonly reported adverse reactions (occurring in ≥10% of patients) that were considered related to VPRIV are shown in Table 2. The most common adverse reactions were infusion-related reactions.

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice.

Table 2: Adverse Reactions Observed in a 10% of Patients with Type 1 Gaucher Disease Treated with VPRIV

System Organ Class Preferred Term	Number of Patients (%)	
	Naïve to ERT N = 54	Switched from Imiglucerase to VPRIV N = 40
Nervous system disorders		
Headache	19 (35.2)	12 (30)
Dizziness	12 (22.2)	3 (7.5)
Gastrointestinal disorders		
Abdominal pain	10 (18.5)	6 (15)
Nausea	3 (5.6)	4 (10)
Musculoskeletal and connective tissue disorders		
Back pain	9 (16.7)	7 (17.5)
Joint pain (limb)	8 (14.8)	3 (7.5)
Infections and infestations		
Upper respiratory tract infection	17 (31.5)	12 (30)
Investigations		
Activated partial thromboplastin time prolonged	6 (11.1)	2 (5)
General disorders and administration site conditions		
Infusion-related reactions*	28 (51.9)	9 (22.5)
Pyrexia	12 (22.2)	5 (12.5)
Asthenia/Fatigue	7 (13)	5 (12.5)

*Describe any event considered related to and occurring within up to 24 hours of VPRIV infusion.

Less common adverse reactions affecting more than one patient (>5% in the treatment-naïve group and >2% in patients switched from imiglucerase to VPRIV treatment) were back pain, body aches, rash, urticaria, flushing, hypotension, and hypertension.

Pediatric Patients

All adult adverse reactions in VPRIV are considered relevant to pediatric patients (ages 4 to 17 years). Adverse reactions more commonly seen in pediatric patients compared to adult patients include (>10% difference): upper respiratory tract infection, rash, PTI, prolonged, and pyruvate.

Immunogenicity

As with all therapeutic proteins, there is a potential for immunogenicity in clinical studies. In 54 treatment-naïve patients treated with VPRIV developed IgG class antibodies to VPRIV. In this patient, the antibodies were determined to be neutralizing in an in vitro assay. The infusion-related reactions were reported for this patient. It is unknown if the presence of IgG antibodies to VPRIV is associated with a higher risk of infusion reactions. Patients with an immune response to other enzyme replacement therapies who are switching to VPRIV should continue to be monitored for antibodies.

Immunogenicity assay results are highly dependent on the sensitivity and specificity of the assay. Additionally, the observed incidence of antibody positivity in an assay may be influenced by several factors, including assay methodology, sample handling, timing of sample collection, concomitant medications, and underlying disease. For these reasons, comparisons of the incidence of antibodies to VPRIV with the incidence of antibodies to other products may be misleading.

7 DRUG INTERACTIONS

No drug-drug interaction studies have been conducted.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy - Category B

Reproduction studies with velaglucease alfa have been performed in pregnant rats at intravenous doses up to 17 mg/kg/day (102 mg/kg/day, about 1.8 times the recommended human dose of 60 Units/kg/day or 55.5 mg/kg/day based on the body surface area). Reproduction studies have been performed in pregnant rabbits at intravenous doses up to 20 mg/kg/day (240 mg/kg/day, about 4.3 times the recommended human dose of 60 Units/kg/day based on the body surface area). These studies did not reveal any evidence of impaired fertility or harm to the fetus due to velaglucease alfa.

A pre- and perinatal development study in rats showed no evidence of any adverse effects on pre- and perinatal development at doses up to 17 mg/kg (102 mg/kg/day, about 1.8 times the recommended human dose of 60 Units/kg/day based on the body surface area). There are, however, no adequate and well-controlled studies in pregnant women. Because animal reproduction studies are not always predictive of human response, VPRIV should be used during pregnancy only if clearly needed.

8.2 Nursing Mothers

There are no data from studies in lactating women. It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when VPRIV is administered to a nursing woman.

8.4 Pediatric Use

The safety and effectiveness of VPRV have been established in patients between 4 and 17 years of age. Use of VPRV in this age group is supported by evidence from adequate and well-controlled studies of VPRV in adults and pediatric (20 of 94 (21%) patients). The safety and efficacy profiles were similar between pediatric and adult patients (see Adverse Reactions (5.1) and Clinical Studies (14)). The safety of VPRV has not been established in pediatric patients younger than 4 years of age.

8.5 Geriatric Use

During clinical studies, 4 patients aged 65 or older were treated with VPRV. Clinical studies of VPRV did not include sufficient numbers of subjects aged 65 and over to determine whether they responded differently from younger subjects. Other reported clinical experience has not identified differences in response between the elderly and younger patients. In general, dose selection for an elderly patient should be approached cautiously, considering potential renal impairment.

10 OVERDOSEAGE

There is no experience with overdose of VPRV.

11 DESCRIPTION

The active ingredient of VPRV is valproic acid, which is produced by gene activation technology in a human fibroblast cell line. Valproic acid is a glycoprotein of 497 amino acids with a molecular weight of approximately 63 kDa. Valproic acid has the same amino acid sequence as the naturally occurring human enzyme, glutathione S-transferase. Valproic acid contains 5 potential N-linked glycosylation sites; four of these sites are occupied by glycan chains. Valproic acid is manufactured to contain predominantly high mannose-type N-linked glycan chains. The high mannose type N-linked glycan chains are specifically recognized and internalized via the mannose receptor present on the surface of macrophages, the cells that accumulate glucosaminoglycans in Gaucher disease. Valproic acid catalyzes the hydrolysis of the glycosylated glucosaminoglycans to glucose and ceramide in the lysosome.

VPRV is stored by Unilever, where one Unit of enzyme activity is defined as the quantity of enzyme required to convert one micromole of p-nitrophenyl 3-O-glucopyranoside to p-nitrophenol per minute at 37°C.

VPRV is supplied as a sterile, preservative free, lyophilized powder in single-use vials. Following reconstitution with Sterile Water for Injection, USP, the solution contains the components listed in Table 3.

Table 3: VPRV Composition Following Reconstitution

	Extractable 200 Units/vial	Extractable 400 Units/vial
Active ingredient		
valproic acid	200 Units	400 Units
Inactive ingredients		
citric acid, monohydrate	2.52 mg	5.04 mg
polyorbital 2D	0.22 mg	0.44 mg
sodium citrate, dihydrate	25.58 mg	51.16 mg
sucrose	100 mg	200 mg

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

Gaucher disease is an autosomal recessive disorder caused by mutations in the GBA gene, which results in a deficiency of the lysosomal enzyme beta-glucuronidase. Glucuronidase catalyzes the conversion of the sphingolipid glucosaminoglycans into glucose and ceramide. The enzymatic deficiency causes an accumulation of glucosaminoglycans in the lysosomal compartment of macrophages, giving rise to foam cells or "Gaucher cells." In this lysosomal storage disorder (LSD), clinical features are reflective of the accumulation of Gaucher cells in the bone, spleen, liver, marrow, and other organs. The accumulation of Gaucher cells in the bone and spleen leads to osteopenia. Features of Gaucher cells in the bone marrow and spleen lead to clinically significant anemia and thrombocytopenia.

Valproic acid catalyzes the hydrolysis of glucosaminoglycans, reducing the amount of accumulated glucosaminoglycans.

12.3 Pharmacokinetics

In a multicenter study conducted in pediatric (107, 4 to 17 years old) and adult (10, 19 to 62 years old) patients with type 1 Gaucher disease, pharmacokinetic evaluations were performed at Weeks 1 and 37 following 60-minute intravenous infusions of VPRV 60 Units/kg every other week. Serum valproic acid concentrations declined rapidly with a mean half-life of 11 to 12 minutes. The mean valproic acid clearance ranged from 0.72 to 1.56 mL/min/kg. The mean volume of distribution at steady state ranged from 82 to 108 mL/kg (0.2% to 0.3% of body weight). However, because an inadequately validated analytical assay method was used in this evaluation, the accurate and reliable pharmacokinetic parameter values are not currently available. No accumulation or change in valproic acid pharmacokinetics over time from Weeks 1 to 37 was observed upon multiple-dosing 60 Units/kg every other week.

Based on the limited data, there were no notable pharmacokinetic differences between male and female patients in this study. The effect of age on pharmacokinetics of valproic acid was inconclusive.

The effect of end-organ activity/formation on the pharmacokinetic parameters of valproic acid is unknown.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

Long-term studies in animals to evaluate carcinogenic potential or studies to evaluate mutagenic potential have not been performed with valproic acid.

In a male and female fertility study in rats, valproic acid did not cause any significant adverse effect on male or female fertility parameters up to a maximum dose of 17 mg/kg/day (102 mg/m²/day, about 1.8 times the recommended human dose of 60 Units/kg/day based on the body surface area).

14 CLINICAL STUDIES

The efficacy of VPRV was assessed in three clinical studies in a total of 89 patients with type 1 Gaucher disease: 82 patients age 4 years and older received VPRV and 17 patients age 3 years and older received imiglucerase. Studies 1 and 2 were conducted in patients who were not currently receiving Gaucher disease-specific therapy. Study 3 was conducted in patients who were receiving imiglucerase treatment immediately before starting VPRV. In these studies, VPRV was administered intravenously over 60 minutes at doses ranging from 15 Units/kg to 60 Units/kg every other week.

14.1 Studies of VPRV as Initial Therapy

Study 1 was a 12-month, randomized, double-blind, parallel-group, multicenter study in 25 patients age 4 years and older with Gaucher disease-related anemia and other thrombocytopenia or organomegaly. Patients were not allowed to have had Gaucher-specific therapy for at least the previous 30 months, but had no prior therapy. The mean age was 25 years and 60% were male. Patients were randomized to receive either VPRV at a dose of either 45 Units/kg (N=13) or 60 Units/kg (N=12) every other week.

At baseline, mean hemoglobin concentration was 13.6 g/dL, mean platelet count was 57 x 10⁹/L, mean liver volume was 3.6% of body weight (5.8%), and mean spleen volume was 2.9% BW. For all studies, liver and spleen volumes were measured by MRI. The changes in clinical parameters after 12 months of treatment are shown in Table 4. The observed change from baseline in the primary endpoint, hemoglobin concentration, was considered to be clinically meaningful in light of the natural history of untreated Gaucher disease.

Table 4: Mean Change from Baseline to Month 12 for Clinical Parameters in Patients with Type 1 Gaucher Disease Initiating Therapy with VPRV in Study 1

Clinical Parameter	Mean Changes from Baseline ± Std. Err. of the Mean	
	VPRV Dose (given every other week)	
	45 Units/kg N = 13	60 Units/kg N = 12
Hemoglobin concentration change (g/dL)	2.4 ± 0.4*	2.4 ± 0.3*
Platelet count change (x 10 ⁹ /L)	41 ± 14*	51 ± 12*
Liver volume change (5.8% BW)	-0.30 ± 0.29	-0.84 ± 0.33
Spleen volume change (5.8% BW)	-1.9 ± 0.6*	-1.9 ± 0.5*

* Primary study endpoint was hemoglobin concentration change in the 60 Units/kg group, p < 0.001

* Statistically significant change from baseline after adjusting for performing multiple tests

Study 2 was a 6-month, randomized, double-blind, active-controlled (imiglucerase) parallel-group, multicenter study in 34 patients age 3 years and older. Patients were required to have Gaucher disease-related anemia and either thrombocytopenia or organomegaly. Patients were not allowed to have had Gaucher-specific therapy for at least the previous 12 months. The mean age was 30 years and 55% were female. The youngest patient who received VPRV was age 4 years. Patients were randomized to receive either 60 Units/kg of VPRV (N=17) or 60 Units/kg of imiglucerase (N=17) every other week.

At baseline, the mean hemoglobin concentration was 11.0 g/dL, mean platelet count was 171 x 10⁹/L, and mean liver volume was 3.5% BW. For the patients who had not had splenectomy (7 in each group) the mean spleen volume was 2.4% BW. After 6 months of treatment, the mean absolute increase from baseline in hemoglobin concentration was 1.6 g/dL (1.0 g/dL) for patients treated with VPRV. The mean treatment difference in change from baseline to 6 months (VPRV - imiglucerase) was 0.1 g/dL (0.4 g/dL). In Studies 1 and 2, examination of age and gender subgroups did not identify differences in response to VPRV among these subgroups. The number of non-Gaucher patients in these studies was too small to adequately assess any difference in effects by race.

14.2 Study in Patients Switching from Imiglucerase Treatment to VPRV

Study 3 was a 12-month, open-label, single-arm, multicenter study in 40 patients age 3 years and older who had been receiving treatment with imiglucerase at doses ranging between 15 Units/kg to 60 Units/kg for a minimum of 30 consecutive months. Patients also were required to have a stable baseline dose of imiglucerase for at least 6 months prior to enrollment. The mean age was 30 years and 55% were female. Imiglucerase therapy was stopped, and treatment with VPRV was administered every other week at the same number of units as the patient's previous imiglucerase dose. Adjustment of dosage was allowed by study criteria if needed in order to maintain clinical parameters.

Hemoglobin concentration and platelet counts remained stable on average through 12 months of VPRV treatment. After 12 months of treatment with VPRV the median hemoglobin concentration was 13.3 g/dL (range: 10.8, 16.1) vs. the baseline value of 13.8 g/dL (range: 10.4, 16.5), and the median platelet count after 12 months was 174 x 10⁹/L (range: 24, 409) vs. the baseline value of 162 x 10⁹/L (range: 25, 395). No patient required dosage adjustment during the 12-month treatment period.

15 REFERENCES

1. Pastores GM, Whitcomb NJ, Aerts H, et al. Therapeutic Goals in the Treatment of Gaucher Disease. *Stem Cells* 2004; 4 (4 Suppl):S4-S14.

16 HOW SUPPLIED/STORAGE AND HANDLING

VPRV is a sterile, preservative free, lyophilized powder requiring reconstitution and further dilution prior to use. It is supplied in individually packaged glass vials, which are closed with a half rubber stopper with a fluorocarbon coating and are sealed with an aluminum crimped with a flip-off plastic cap. The vials are intended for single use only. VPRV is available as: 200 Units/vial NDC 54002-701-02 and 400 Units/vial NDC 54002-701-04.

16.1 Storage

VPRV should be stored in a refrigerator at 2 to 8°C (36 to 46°F). Do not use VPRV after the expiration date on the vial.

Do not freeze.

Protect vial from light.

17 PATIENT COUNSELING INFORMATION

VPRV should be administered under the supervision of a healthcare professional. VPRV is a treatment that is given intravenously (by IV) every other week. The infusion typically takes up to 60 minutes.

Patients should be advised that VPRV may cause hypersensitivity reactions or infusion-related reactions. Infusion-related reactions can usually be managed by slowing the infusion rate, treatment with medications such as antihistamines, antipyretics and/or corticosteroids, and/or stopping and resuming treatment with increased infusion time. Pre-treatment with antihistamines and/or corticosteroids may prevent subsequent reactions. Treatment with VPRV should be carefully re-evaluated if significant evidence of hypersensitivity to the product occurs (see Warnings and Precautions (5.1, 5.2)).

18 Only

VPRV is manufactured by:

Shire Human Genetic Therapies, Inc.
700 Main Street
Cambridge, MA 02139

OnePact is a service mark and VPRV is a trademark of Shire Human Genetic Therapies, Inc.

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment C

NDA approval letter from FDA to Shire Human Genetic Therapies, Inc., dated
February 26, 2010 (with enclosure)



DEPARTMENT OF HEALTH AND HUMAN SERVICES

Food and Drug Administration
Silver Spring MD 20993

NDA 022575

NDA APPROVAL

Shire Human Genetic Therapies, Inc.
Attention: Nikhil Mehta, Ph.D.
Vice President, Global Regulatory Affairs
700 Main Street
Cambridge, MA 02139

Dear Dr. Mehta:

Please refer to your new drug application (NDA) dated August 31, 2009, received August 31, 2009, submitted under section 505(b) of the Federal Food, Drug, and Cosmetic Act for VPRIV (velaglucerase alfa for injection).

We acknowledge receipt of your submissions dated July 30, August 31, September 17, 22, and 28, October 1, 9, 12, 23, and 29, November 16 and 20, December 1, 4, 15, 18, 22, and 31, 2009, and January 13, 14, 15, 26, and 27, and February 1, 8, 9, 17, 19, and 25, 2010.

This new drug application provides for the use of VPRIV (velaglucerase alfa for injection) for long-term enzyme replacement therapy (ERT) for pediatric and adult patients with type 1 Gaucher disease.

We have completed our review of this application, as amended. It is approved, effective on the date of this letter, for use as recommended in the enclosed agreed-upon labeling text.

Your application was not referred to an advisory committee because this drug is not the first in its class, the clinical study design was acceptable, the application did not raise significant safety or efficacy issues, the application did not raise significant public health questions on the role of the drug in the diagnosis, cure, mitigation, treatment or prevention of a disease, and outside expertise was not necessary.

CONTENT OF LABELING

As soon as possible, but no later than 14 days from the date of this letter, please submit the content of labeling [21 CFR 314.50(l)] in structured product labeling (SPL) format, as described at <http://www.fda.gov/ForIndustry/DataStandards/StructuredProductLabeling/default.htm>, that is identical to the submitted labeling (package insert submitted February 25, 2010). For administrative purposes, please designate this submission, "SPL for approved NDA 022575."

CARTON AND IMMEDIATE CONTAINER LABELS

We acknowledge your February 19, 2010, submission containing final printed carton and container labels.

Marketing the product with final printed labeling that is not identical to the approved labeling text may render the product misbranded and an unapproved new drug.

REQUIRED PEDIATRIC ASSESSMENTS

Under the Pediatric Research Equity Act (PREA) (21 U.S.C. 355c), all applications for new active ingredients, new indications, new dosage forms, new dosing regimens, or new routes of administration are required to contain an assessment of the safety and effectiveness of the product for the claimed indication in pediatric patients unless this requirement is waived, deferred, or inapplicable.

Because this drug product for this indication has an orphan drug designation, you are exempt from this requirement.

POSTMARKETING COMMITMENTS SUBJECT TO REPORTING REQUIREMENTS UNDER SECTION 506B

We remind you of your postmarketing commitments in your submission dated February 25, 2010. These commitments are listed below.

- 1600-01 Shire commits to utilize an antibody screening cut point based on a mean + 1.645 standard deviation for assay values from treatment naïve Gaucher patients. Shire will utilize the same methodology to calculate the anti-imiglucerase ECL cut point.

Final Report Submission: May 31, 2010

- 1600-02 Shire commits to revise the cut point for the confirmatory anti-velaglucerase and anti-imiglucerase screening assays to a level that is less than or equal to the cut point of the screening assay.

Final Report Submission: May 31, 2010

- 1600-03 Shire commits to re-assess the IgE cut point for the current ECL methodology using a chemically synthesized hybrid control. Shire commits to support assay validation using patient baseline values.

Final Report Submission: May 31, 2010

- 1600-04 Shire commits to develop an assay to measure the ability of patient antibodies to block the uptake of velaglucerase and imiglucerase into target cells.

Final Report Submission: November 30, 2010

- 1600-05 Shire commits to re-analyze all archived pharmacokinetic (PK) samples for Study TKT032 (using adequate in-process quality controls and standard curves) and recalculate velaglucerase alfa PK parameters.

Study Completion Date: May 31, 2010
Final Report Submission: June 30, 2010

- 1600-06 Shire commits to conduct a prospective PK study in patients with Type 1 Gaucher disease in the case that Shire fails to adequately characterize velaglucerase alfa PK using the archived PK samples (discussed under PMC #1600-05 above).

Final Protocol Submission: December 31, 2010
Study Completion Date: March 31, 2013
Final Report Submission: September 30, 2013

POSTMARKETING COMMITMENTS NOT SUBJECT TO THE REPORTING REQUIREMENTS UNDER SECTION 506B

We remind you of your postmarketing commitments in your submission dated February 25, 2010. These commitments are listed below.

- 1600-07 Shire commits to develop and implement a kinetic assay with a physiologically relevant substrate for drug substance and drug product release and stability testing. Results and specifications will be included in the final report.

Final Report Submission: December 31, 2011

- 1600-08 Shire commits to develop and implement a quantitative method that measures total carbohydrate content. Results and specifications will be included in the final report.

Final Report Submission: February 28, 2011

- 1600-09 Shire commits to replace the non-quantitative SDS-PAGE Silver stain method with a quantitative SDS-PAGE Coomassie test. Results and specifications will be included in the final report.

Final Report Submission: February 28, 2011

- 1600-10 Shire commits to demonstrate that Long R3 IGF1 is well controlled to ensure no impact on product quality. The results will be included in the final report.

Final Report Submission: February 28, 2011

- 1600-11 Shire commits to demonstrate the clearance capability of the process to remove hydrocortisone through hydrocortisone spike studies. The results will be included in the final report.

Final Report Submission: November 30, 2010

- 1600-12 Shire commits to re-evaluate drug substance and drug product release and stability specifications. Shire will submit the revised specifications and supporting data in the final report.

Final Report Submission: December 31, 2011

- 1600-13 Shire commits to update the specifications for SEC, RP-HPLC, and the glycan map, and to include acceptance criteria for the leading shoulder in SEC-HPLC, for peaks A and B in RP-HPLC, and for peak group 2 in the glycan map.

Final Report Submission: July 1, 2010

- 1600-14 Shire commits to update the peptide map specification using new acceptance criteria to reflect control of impurities. Shire commits to add the peptide map as a drug substance and drug product release and stability test with the new acceptance criteria.

Final Report Submission: July 1, 2010

- 1600-15 Shire commits to include the cellular uptake bioassay for drug product release testing.

Final Report Submission: April 1, 2010

- 1600-16 Shire commits to provide a report containing the sub-visible particulates (2 – 10 μ m) analyses, risk assessment and risk mitigation strategies.

Final Report Submission: September 30, 2010

- 1600-17 Shire commits to include drug substance and drug product stress conditions in the annual stability program. The revised stability protocols will be included.

Final Protocol Submission: April 1, 2010

- 1600-18 Shire commits to evaluate the impact of pH on the in-use stability of the drug product and to provide assurance that procedures are in place to control this risk to product quality.

Final Protocol Submission: December 31, 2010

Submit clinical protocols to your IND 061220 for this product. Submit nonclinical and chemistry, manufacturing, and controls protocols and all study final reports to this NDA. In addition, under 21 CFR 314.81(b)(2)(vii) and 314.81(b)(2)(viii) you should include a status summary of each commitment in your annual report to this NDA. The status summary should include expected summary completion and final report submission dates, any changes in plans since the last annual report, and, for clinical studies/trials, number of patients entered into each study/trial. All submissions, including supplements, relating to these postmarketing commitments should be prominently labeled "Postmarketing Commitment Protocol," "Postmarketing Commitment Final Report," or "Postmarketing Commitment Correspondence."

PROMOTIONAL MATERIALS

You may request advisory comments on proposed introductory advertising and promotional labeling. To do so, submit, in triplicate, a cover letter requesting advisory comments, the proposed materials in draft or mock-up form with annotated references, and the package insert to:

Food and Drug Administration
Center for Drug Evaluation and Research
Division of Drug Marketing, Advertising, and Communications
5901-B Ammendale Road
Beltsville, MD 20705-1266

As required under 21 CFR 314.81(b)(3)(i), you must submit final promotional materials, and the package insert, at the time of initial dissemination or publication, accompanied by a Form FDA 2253. For instruction on completing the Form FDA 2253, see page 2 of the Form. For more information about submission of promotional materials to the Division of Drug Marketing, Advertising, and Communications (DDMAC), see <http://www.fda.gov/AboutFDA/CentersOffices/CDER/ucm090142.htm>.

Please submit one market package of the drug product when it is available.

LETTERS TO HEALTH CARE PROFESSIONALS

If you issue a letter communicating important safety-related information about this drug product (i.e., a "Dear Health Care Professional" letter), we request that you submit an electronic copy of the letter to both this NDA and to the following address:

MedWatch
Food and Drug Administration
Suite 12B-05
5600 Fishers Lane
Rockville, MD 20857

REPORTING REQUIREMENTS

We remind you that you must comply with reporting requirements for an approved NDA (21 CFR 314.80 and 314.81).

MEDWATCH-TO-MANUFACTURER PROGRAM

The MedWatch-to-Manufacturer Program provides manufacturers with copies of serious adverse event reports that are received directly by the FDA. New molecular entities and important new biologics qualify for inclusion for three years after approval. Your firm is eligible to receive copies of reports for this product. To participate in the program, please see the enrollment instructions and program description details at

<http://www.fda.gov/Safety/MedWatch/HowToReport/ucm166910.htm>.

If you have any questions, call Wes Ishihara, Regulatory Project Manager, at (301) 796-0069.

Sincerely,

{See appended electronic signature page}

Julie Beitz, M.D.

Director

Office of Drug Evaluation III

Center for Drug Evaluation and Research

Enclosure: Package Insert

HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use VPRIV safely and effectively. See full prescribing information for VPRIV.

VPRIV™ (velaglucerase alfa for injection)

Initial U.S. Approval: 2010

INDICATIONS AND USAGE

VPRIV (velaglucerase alfa for injection) is a hydrolytic lysosomal glucocerebroside-specific enzyme indicated for long-term enzyme replacement therapy (ERT) for pediatric and adult patients with type 1 Gaucher disease (1).

DOSAGE AND ADMINISTRATION

- 60 Units/kg administered every other week as a 60-minute intravenous infusion (2).
- Patients currently being treated with imiglucerase for Gaucher disease can be switched to VPRIV. Patients previously treated on a stable dose of imiglucerase are recommended to begin treatment with VPRIV at that same dose when they switch from imiglucerase to VPRIV (2).
- Physicians can make dosage adjustments based on achievement and maintenance of each patient's therapeutic goals. Clinical trials have evaluated doses ranging from 15 Units/kg to 60 Units/kg every other week (2).

DOSAGE FORMS AND STRENGTHS

- Lyophilized powder to be reconstituted and diluted for infusion (3).
- Available in 200 Units and 400 Units single-use vials (3).

CONTRAINDICATIONS

- None (4).

WARNINGS AND PRECAUTIONS

- Hypersensitivity reactions: Treatment with VPRIV should be carefully re-evaluated in the presence of significant evidence of hypersensitivity to the product (5.1).
- Infusion-related reactions (5.2).

ADVERSE REACTIONS

- The most common adverse reactions during clinical studies were infusion-related reactions (5.2, 6.1).
- Other commonly observed adverse reactions in $\geq 10\%$ of patients were: headache, dizziness, abdominal pain, nausea, back pain, joint pain, upper respiratory tract infection, activated PTT prolonged, fatigue/asthenia, and pyrexia (6.1).

To report SUSPECTED ADVERSE REACTIONS, contact Shire Human Genetic Therapies, Inc. at the OnePathSM phone # 1-866-888-0660 or MedInfoGlobal@Shire.com, or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch

See Section 17 for PATIENT COUNSELING INFORMATION.

Revised: 02/2010

FULL PRESCRIBING INFORMATION: CONTENTS*

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*Sections or subsections omitted from the full prescribing information are not listed.

VPRIV™ (velaglucerase alfa for injection)

FULL PRESCRIBING INFORMATION

1 INDICATIONS AND USAGE

VPRIV (velaglucerase alfa for injection) is a hydrolytic lysosomal glucocerebroside-specific enzyme indicated for long-term enzyme replacement therapy (ERT) for pediatric and adult patients with type 1 Gaucher disease.

2 DOSAGE AND ADMINISTRATION

2.1 Recommended Dose

The recommended dose is 60 Units/kg administered every other week as a 60-minute intravenous infusion.

Patients currently being treated with imiglucerase for type 1 Gaucher disease may be switched to VPRIV. Patients previously treated on a stable dose of imiglucerase are recommended to begin treatment with VPRIV at that same dose when they switch from imiglucerase to VPRIV.

Dosage adjustments can be made based on achievement and maintenance of each patient's therapeutic goals. Clinical studies have evaluated doses ranging from 15 Units/kg to 60 Units/kg every other week.

VPRIV should be administered under the supervision of a healthcare professional.

2.2 Preparation and Administration Instructions

Use aseptic technique

VPRIV is a lyophilized powder, which requires reconstitution and dilution, and is intended for intravenous infusion only. VPRIV contains no preservatives and vials are single-use only. Discard any unused solution. VPRIV should be prepared as follows:

Determine the number of vials to be reconstituted based on the individual patient's weight and the prescribed dose. Follow the instructions in Table 1 for reconstitution.

Table 1: Reconstitution Instructions

	200 Units/vial	400 Units/vial
Volume of Sterile Water for Injection, USP, for reconstitution	2.2 mL	4.3 mL
Concentration after reconstitution	100 Units/mL	100 Units/mL
Withdrawal volume	2 mL	4 mL

Upon reconstitution, mix vials gently. DO NOT SHAKE. Prior to further dilution, visually inspect the solution in the vials; the solution should be clear to slightly opalescent and colorless; do not use if the solution is discolored or if foreign particulate matter is present. Withdraw the calculated volume of drug from the appropriate number of vials and dilute the total volume required in 100 mL of 0.9% sodium chloride solution suitable for IV administration. Mix gently. DO NOT SHAKE.

VPRIV should be administered over 60 minutes. VPRIV should not be infused with other products in the same infusion tubing as the compatibility in solution with other products has not been evaluated. The diluted solution should be filtered through an in-line low protein-binding 0.2µm filter during administration.

As VPRIV contains no preservatives, once reconstituted the product should be used immediately. If immediate use is not possible, the reconstituted or diluted product may be stored for up to 24 hours at 2 to 8°C (36 to 46°F). Do not freeze. Protect from light. The infusion should be completed within 24 hours of reconstitution of vials.

3 DOSAGE FORMS AND STRENGTHS

VPRIV is a sterile, white to off-white, lyophilized powder for reconstitution with Sterile Water for Injection, USP, to yield a final concentration of 100 Units/mL.

VPRIV is available as 200 Units and 400 Units single-use vials.

4 CONTRAINDICATIONS

None.

5 WARNINGS AND PRECAUTIONS

5.1 Hypersensitivity Reactions

Hypersensitivity reactions have been reported in patients in clinical studies with VPRIV [*see Adverse Reactions (6.1)*]. As with any intravenous protein product, hypersensitivity reactions are possible, therefore appropriate medical support should be readily available when VPRIV is administered. If a severe reaction occurs, current medical standards for emergency treatment are to be followed.

Treatment with VPRIV should be approached with caution in patients who have exhibited symptoms of hypersensitivity to the active ingredient or excipients in the drug product or to other enzyme replacement therapy.

5.2 Infusion-related Reactions

Infusion-related reactions were the most commonly observed adverse reactions in patients treated with VPRIV in clinical studies. The most commonly observed symptoms of infusion-related reactions were: headache, dizziness, hypotension, hypertension, nausea, fatigue/asthenia, and pyrexia. Generally the infusion-related reactions were mild and, in treatment-naïve patients, onset occurred mostly during the first 6 months of treatment and tended to occur less frequently

with time.

The management of infusion-related reactions should be based on the severity of the reaction, e.g. slowing the infusion rate, treatment with medications such as antihistamines, antipyretics and/or corticosteroids, and/or stopping and resuming treatment with increased infusion time.

Pre-treatment with antihistamines and/or corticosteroids may prevent subsequent reactions in those cases where symptomatic treatment was required. Patients were not routinely pre-medicated prior to infusion of VPRIV during clinical studies.

6 ADVERSE REACTIONS

6.1 Clinical Studies Experience

The data described below reflect exposure of 94 patients with type 1 Gaucher disease who received VPRIV at doses ranging from 15 Units/kg to 60 Units/kg every other week in 5 clinical studies. Fifty-four (54) patients were naïve to ERT and received VPRIV for 9 months and 40 patients switched from imiglucerase to VPRIV treatment and received VPRIV for 12 months [see *Clinical Studies (14)*]. Patients were between 4 and 71 years old at time of first treatment with VPRIV, and included 46 male and 48 female patients.

The most serious adverse reactions in patients treated with VPRIV were hypersensitivity reactions [see *Warnings and Precautions (5.1)*].

The most commonly reported adverse reactions (occurring in $\geq 10\%$ of patients) that were considered related to VPRIV are shown in Table 2. The most common adverse reactions were infusion-related reactions.

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice.

Table 2: Adverse Reactions Observed in $\geq 10\%$ of Patients with Type 1 Gaucher Disease Treated with VPRIV

System Organ Class Preferred Term	Naïve to ERT N = 54	Switched from imiglucerase to VPRIV N = 40
	Number of Patients (%)	
Nervous system disorders		
Headache	19 (35.2)	12 (30)
Dizziness	12 (22.2)	3 (7.5)
Gastrointestinal disorders		
Abdominal pain	10 (18.5)	6 (15)
Nausea	3 (5.6)	4 (10)
Musculoskeletal and connective tissue disorders		
Back pain	9 (16.7)	7 (17.5)
Joint pain (knee)	8 (14.8)	3 (7.5)
Infections and infestations		
Upper respiratory tract infection	17 (31.5)	12 (30)
Investigations		
Activated partial thromboplastin time prolonged	6 (11.1)	2 (5)
General disorders and administration site conditions		
Infusion-related reaction*	28 (51.9)	9 (22.5)
Pyrexia	12 (22.2)	5 (12.5)
Asthenia/Fatigue	7 (13)	5 (12.5)
*Denotes any event considered related to and occurring within up to 24 hours of VPRIV infusion		

Less common adverse reactions affecting more than one patient ($>3\%$ in the treatment-naïve group and $>2\%$ in patients switched from imiglucerase to VPRIV treatment) were bone pain, tachycardia, rash, urticaria, flushing, hypertension, and hypotension.

Pediatric Patients

All adult adverse reactions to VPRIV are considered relevant to pediatric patients (ages 4 to 17 years). Adverse reactions more commonly seen in pediatric patients compared to adult patients include ($>10\%$ difference): upper respiratory tract infection, rash, aPTT prolonged, and pyrexia.

Immunogenicity

As with all therapeutic proteins, there is a potential for immunogenicity. In clinical studies, 1 of 54 treatment-naïve patients treated with VPRIV developed IgG class antibodies to VPRIV.

In this patient, the antibodies were determined to be neutralizing in an in vitro assay. No infusion-related reactions were reported for this patient. It is unknown if the presence of IgG antibodies to VPRIV is associated with a higher risk of infusion reactions. Patients with an immune response to other enzyme replacement therapies who are switching to VPRIV should continue to be monitored for antibodies.

Immunogenicity assay results are highly dependent on the sensitivity and specificity of the assay. Additionally, the observed incidence of antibody positivity in an assay may be influenced by several factors, including assay methodology, sample handling, timing of sample collection, concomitant medications, and underlying disease. For these reasons, comparison of the incidence of antibodies to VPRIV with the incidence of antibodies to other products may be misleading.

7 DRUG INTERACTIONS

No drug-drug interaction studies have been conducted.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy – Category B

Reproduction studies with velaglucerase alfa have been performed in pregnant rats at intravenous doses up to 17 mg/kg/day (102 mg/m²/day, about 1.8 times the recommended human dose of 60 Units/kg/day or 1.5 mg/kg/day or 55.5 mg/m²/day based on the body surface area). Reproduction studies have been performed in pregnant rabbits at intravenous doses up to 20 mg/kg/day (240 mg/m²/day, about 4.3 times the recommended human dose of 60 Units/kg/day based on the body surface area). These studies did not reveal any evidence of impaired fertility or harm to the fetus due to velaglucerase alfa.

A pre- and postnatal development study in rats showed no evidence of any adverse effect on pre- and postnatal development at doses up to 17 mg/kg (102 mg/m²/day, about 1.8 times the recommended human dose of 60 Units/kg/day based on the body surface area). There are, however, no adequate and well-controlled studies in pregnant women. Because animal reproduction studies are not always predictive of human response, VPRIV should be used during pregnancy only if clearly needed.

8.3 Nursing Mothers

There are no data from studies in lactating women. It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when VPRIV is administered to a nursing woman.

8.4 Pediatric Use

The safety and effectiveness of VPRIV have been established in patients between 4 and 17 years of age. Use of VPRIV in this age group is supported by evidence from adequate and well-controlled studies of VPRIV in adults and pediatric [20 of 94 (21%)] patients. The safety and efficacy profiles were similar between pediatric and adult patients [*see Adverse Reactions (6.1) and Clinical Studies (14)*]. The safety of VPRIV has not been established in pediatric patients

younger than 4 years of age.

8.5 Geriatric Use

During clinical studies 4 patients aged 65 or older were treated with VPRIV. Clinical studies of VPRIV did not include sufficient numbers of subjects aged 65 and over to determine whether they respond differently from younger subjects. Other reported clinical experience has not identified differences in responses between the elderly and younger patients. In general, dose selection for an elderly patient should be approached cautiously, considering potential comorbid conditions.

10 OVERDOSAGE

There is no experience with overdose of VPRIV.

11 DESCRIPTION

The active ingredient of VPRIV is velaglucerase alfa, which is produced by gene activation technology in a human fibroblast cell line. Velaglucerase alfa is a glycoprotein of 497 amino acids; with a molecular weight of approximately 63 kDa. Velaglucerase alfa has the same amino acid sequence as the naturally occurring human enzyme, glucocerebrosidase. Velaglucerase alfa contains 5 potential N-linked glycosylation sites; four of these sites are occupied by glycan chains. Velaglucerase alfa is manufactured to contain predominantly high mannose-type N-linked glycan chains. The high mannose type N-linked glycan chains are specifically recognized and internalized via the mannose receptor present on the surface on macrophages, the cells that accumulate glucocerebroside in Gaucher disease. Velaglucerase alfa catalyzes the hydrolysis of the glycolipid glucocerebroside to glucose and ceramide in the lysosome.

VPRIV is dosed by Units/kg, where one Unit of enzyme activity is defined as the quantity of enzyme required to convert one micromole of p-nitrophenyl β -D-glucopyranoside to p-nitrophenol per minute at 37°C.

VPRIV is supplied as a sterile, preservative free, lyophilized powder in single-use vials. Following reconstitution with Sterile Water for Injection, USP, the solution contains the components listed in Table 3.

Table 3: VPRIV Composition Following Reconstitution

	Extractable 200 Units/vial	Extractable 400 Units/vial
Active Ingredient		
velaglucerase alfa	200 Units	400 Units
Inactive Ingredients		
citric acid, monohydrate	2.52 mg	5.04 mg
polysorbate 20	0.22 mg	0.44 mg
sodium citrate, dihydrate	25.88 mg	51.76 mg
sucrose	100 mg	200 mg

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

Gaucher disease is an autosomal recessive disorder caused by mutations in the GBA gene, which results in a deficiency of the lysosomal enzyme beta-glucocerebrosidase. Glucocerebrosidase catalyzes the conversion of the sphingolipid glucocerebroside into glucose and ceramide. The enzymatic deficiency causes an accumulation of glucocerebroside primarily in the lysosomal compartment of macrophages, giving rise to foam cells or "Gaucher cells". In this lysosomal storage disorder (LSD), clinical features are reflective of the accumulation of Gaucher cells in the liver, spleen, bone marrow, and other organs. The accumulation of Gaucher cells in the liver and spleen leads to organomegaly. Presence of Gaucher cells in the bone marrow and spleen lead to clinically significant anemia and thrombocytopenia.

Velaglucerase alfa catalyzes the hydrolysis of glucocerebroside, reducing the amount of accumulated glucocerebroside.

12.3 Pharmacokinetics

In a multicenter study conducted in pediatric (N=7, 4 to 17 years old) and adult (N=15, 19 to 62 years old) patients with type 1 Gaucher disease, pharmacokinetic evaluations were performed at Weeks 1 and 37 following 60-minute intravenous infusions of VPRIV 60 Units/kg every other week. Serum velaglucerase alfa concentrations declined rapidly with a mean half life of 11 to 12 minutes. The mean velaglucerase alfa clearance ranged from 6.72 to 7.56 mL/min/kg. The mean volume of distribution at steady state ranged from 82 to 108 mL/kg (8.2% to 10.8% of body weight). However, because an inadequately validated analytical assay method was used in the evaluations, the accurate and definitive pharmacokinetic parameter values are not currently available.

No accumulation or change in velaglucerase alfa pharmacokinetics over time from Weeks 1 to 37 was observed upon multiple-dosing 60 Units/kg every other week.

Based on the limited data, there were no notable pharmacokinetic differences between male and female patients in this study. The effect of age on pharmacokinetics of velaglucerase alfa was inconclusive.

The effect of anti-drug antibody formation on the pharmacokinetic parameters of velaglucerase alfa is unknown.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

Long-term studies in animals to evaluate carcinogenic potential or studies to evaluate mutagenic potential have not been performed with velaglucerase alfa.

In a male and female fertility study in rats, velaglucerase alfa did not cause any significant adverse effect on male or female fertility parameters up to a maximum dose of 17 mg/kg/day (102 mg/m²/day, about 1.8 times the recommended human dose of 60 Units/kg/day based on the

body surface area).

14 CLINICAL STUDIES

The efficacy of VPRIV was assessed in three clinical studies in a total of 99 patients with type 1 Gaucher disease: 82 patients age 4 years and older received VPRIV and 17 patients age 3 years and older received imiglucerase. Studies I and II were conducted in patients who were not currently receiving Gaucher disease-specific therapy. Study III was conducted in patients who were receiving imiglucerase treatment immediately before starting VPRIV. In these studies, VPRIV was administered intravenously over 60 minutes at doses ranging from 15 Units/kg to 60 Units/kg every other week.

14.1 Studies of VPRIV as Initial Therapy

Study I was a 12-month, randomized, double-blind, parallel-dose-group, multinational study in 25 patients age 4 years and older with Gaucher disease-related anemia and either thrombocytopenia or organomegaly. Patients were not allowed to have had disease-specific therapy for at least the previous 30 months; all but one had no prior therapy. The mean age was 26 years and 60% were male. Patients were randomized to receive VPRIV at a dose of either 45 Units/kg (N=13) or 60 Units/kg (N=12) every other week.

At baseline, mean hemoglobin concentration was 10.6 g/dL, mean platelet count was $97 \times 10^9/L$, mean liver volume was 3.6 % of body weight (% BW), and mean spleen volume was 2.9 % BW. For all studies, liver and spleen volumes were measured by MRI. The changes in clinical parameters after 12 months of treatment are shown in Table 4. The observed change from baseline in the primary endpoint, hemoglobin concentration, was considered to be clinically meaningful in light of the natural history of untreated Gaucher disease.

Table 4: Mean Change from Baseline to Month 12 for Clinical Parameters in Patients with Type 1 Gaucher Disease Initiating Therapy with VPRIV in Study I

Clinical Parameter	Mean Changes from Baseline \pm Std. Err. of the Mean	
	VPRIV Dose (given every other week)	
	45 Units/kg N = 13	60 Units/kg N = 12
Hemoglobin concentration change (g/dL)	$2.4 \pm 0.4^*$	$2.4 \pm 0.3^{**}$
Platelet count change ($\times 10^9/L$)	$41 \pm 14^*$	$51 \pm 12^*$
Liver volume change (% BW)	-0.30 ± 0.29	-0.84 ± 0.33
Spleen volume change (% BW)	$-1.9 \pm 0.6^*$	$-1.9 \pm 0.5^*$

** Primary study endpoint was hemoglobin concentration change in the 60 Unit/kg group, $p < 0.001$

* Statistically significant changes from baseline after adjusting for performing multiple tests

Study II was a 9-month, randomized, double-blind, active-controlled (imiglucerase), parallel-group, multinational study in 34 patients age 3 years and older. Patients were required to have Gaucher disease-related anemia and either thrombocytopenia or organomegaly. Patients were

not allowed to have had disease-specific therapy for at least the previous 12 months. The mean age was 30 years and 53% were female; the youngest patient who received VPRIV was age 4 years. Patients were randomized to receive either 60 Units/kg of VPRIV (N=17) or 60 Units/kg of imiglucerase (N=17) every other week.

At baseline, the mean hemoglobin concentration was 11.0 g/dL, mean platelet count was $171 \times 10^9/L$, and mean liver volume was 4.3 % BW. For the patients who had not had splenectomy (7 in each group) the mean spleen volume was 3.4 % BW. After 9 months of treatment, the mean absolute increase from baseline in hemoglobin concentration was $1.6 \text{ g/dL} \pm 0.2 \text{ (SE)}$ for patients treated with VPRIV. The mean treatment difference in change from baseline to 9 months [VPRIV – imiglucerase] was $0.1 \text{ g/dL} \pm 0.4 \text{ (SE)}$.

In Studies I and II, examination of age and gender subgroups did not identify differences in response to VPRIV among these subgroups. The number of non-Caucasian patients in these studies was too small to adequately assess any difference in effects by race.

14.2 Study in Patients Switching from Imiglucerase Treatment to VPRIV

Study III was a 12-month, open-label, single-arm, multinational study in 40 patients age 9 years and older who had been receiving treatment with imiglucerase at doses ranging between 15 Units/kg to 60 Units/kg for a minimum of 30 consecutive months. Patients also were required to have a stable biweekly dose of imiglucerase for at least 6 months prior to enrollment. The mean age was 36 years and 55% were female. Imiglucerase therapy was stopped, and treatment with VPRIV was administered every other week at the same number of units as the patient's previous imiglucerase dose. Adjustment of dosage was allowed by study criteria if needed in order to maintain clinical parameters.

Hemoglobin concentrations and platelet counts remained stable on average through 12 months of VPRIV treatment. After 12 months of treatment with VPRIV the median hemoglobin concentration was 13.5 g/dL (range: 10.8, 16.1) vs. the baseline value of 13.8 g/dL (range: 10.4, 16.5), and the median platelet count after 12 months was $174 \times 10^9/L$ (range: 24, 408) vs. the baseline value of $162 \times 10^9/L$ (range: 29, 399). No patient required dosage adjustment during the 12-month treatment period.

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16 HOW SUPPLIED/STORAGE AND HANDLING

VPRIV is a sterile, preservative free, lyophilized powder requiring reconstitution and further dilution prior to use. It is supplied in individually packaged glass vials, which are closed with a butyl rubber stopper with a fluoro-resin coating and are sealed with an aluminum overseal with a flip-off plastic cap. The vials are intended for single use only. VPRIV is available as: 200 Units/vial NDC 54092-701-02 and 400 Units/vial NDC 54092-701-04.

16.1 Storage

VPRIV should be stored in a refrigerator at 2 to 8°C (36 to 46°F). Do not use VPRIV after the expiration date on the vial. Do not freeze.

Protect vial from light.

17 PATIENT COUNSELING INFORMATION

- VPRIV should be administered under the supervision of a healthcare professional. VPRIV is a treatment that is given intravenously (by IV) every other week. The infusion typically takes up to 60 minutes.
- Patients should be advised that VPRIV may cause hypersensitivity reactions or infusion-related reactions. Infusion-related reactions can usually be managed by slowing the infusion rate, treatment with medications such as antihistamines, antipyretics and/or corticosteroids, and/or stopping and resuming treatment with increased infusion time. Pre-treatment with antihistamines and/or corticosteroids may prevent subsequent reactions. Treatment with VPRIV should be carefully re-evaluated in the presence of significant evidence of hypersensitivity to the product [*see Warnings and Precautions (5.1, 5.2)*].

Rx Only

VPRIV is manufactured by:

Shire Human Genetic Therapies, Inc.
700 Main Street
Cambridge, MA 02139

OnePath is a service mark and VPRIV is a trademark of Shire Human Genetic Therapies, Inc.

Application
Type/Number

Submission
Type/Number

Submitter Name

Product Name

NDA-22575

ORIG-1

SHIRE HUMAN
GENETIC
THERAPIES INC

VELAGLUCERASE ALFA

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/s/

JULIE G BEITZ
02/26/2010

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment D

U.S. Patent No. 7,138,262 B1



US007138262B1

(12) **United States Patent**
Daniel

(10) **Patent No.:** **US 7,138,262 B1**
(45) **Date of Patent:** **Nov. 21, 2006**

(54) **HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH MANNOSE PROTEINS**

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(*) **Notice:** Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) **Appl. No.:** 09/641,471

(22) **Filed:** Aug. 18, 2000

(51) **Int. Cl.**

C12N 9/26 (2006.01)

C12P 19/28 (2006.01)

C12N 5/02 (2006.01)

C12N 5/06 (2006.01)

C12N 15/67 (2006.01)

C07H 21/04 (2006.01)

(52) **U.S. Cl.** 435/201; 435/85; 435/325; 435/358; 435/463; 536/23.2

(58) **Field of Classification Search** 435/201, 435/325, 68.1, 69.1, 72, 85, 358, 365, 463; 536/23.2

See application file for complete search history.

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(57) **ABSTRACT**

The invention features a method of producing a high mannose glucocerebrosidase (hmGCB) which includes: providing a cell which is capable of expressing glucocerebrosidase (GCB), and allowing production of GCB having a precursor oligosaccharide under conditions which prevent the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB, to thereby produce an hmGCB preparation. Preferably, the condition which prevents the removal of at least one mannose residue distal to the pentasaccharide core is inhibition of a class 1 processing mannosidase and/or a class 2 processing mannosidase. The invention also features an hmGCB preparation and methods of using an hmGCB preparation.

63 Claims, 1 Drawing Sheet

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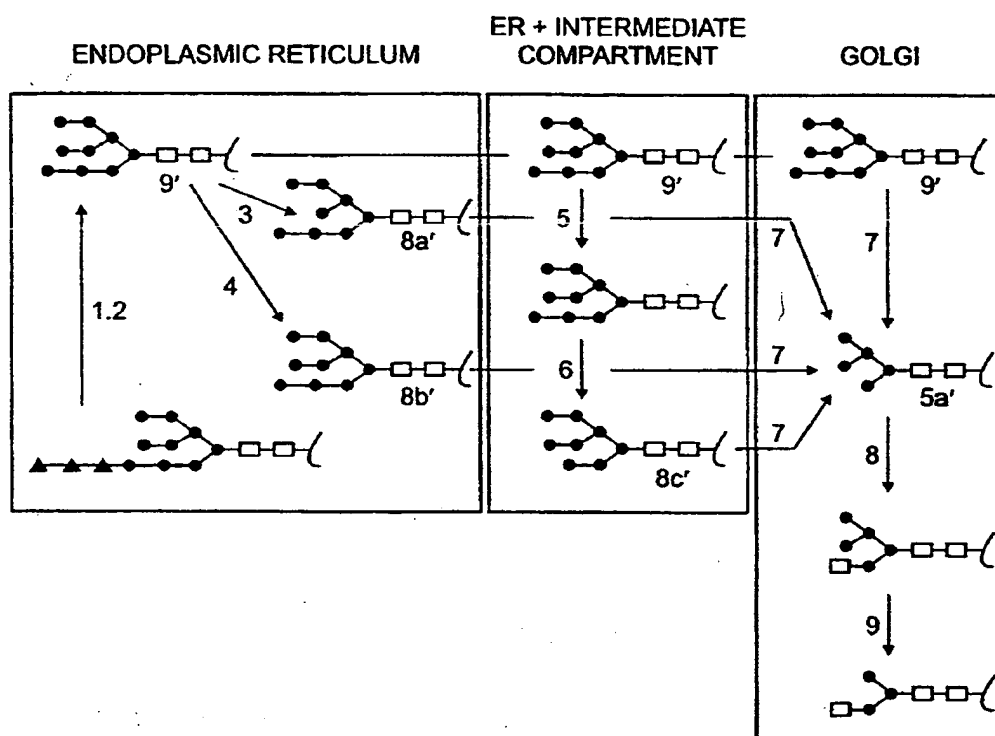


FIG. 1

HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH MANNOSE PROTEINS

BACKGROUND OF THE INVENTION

Gaucher disease is an autosomal recessive lysosomal storage disorder characterized by a deficiency in the lysosomal enzyme, glucocerebrosidase (GCB). GCB hydrolyzes the glycolipid glucocerebroside that is formed after degradation of glycosphingolipids in the membranes of white blood cells and red blood cells. The deficiency in this enzyme causes glucocerebroside to accumulate in large quantities in the lysosomes of phagocytic cells located in the liver, spleen and bone marrow of Gaucher patients. Accumulation of these molecules causes a range of clinical manifestations including splenomegaly, hepatomegaly, skeletal disorder, thrombocytopenia and anemia. (Beutler et al. Gaucher disease; In: The Metabolic and Molecular Bases of Inherited Disease (McGraw-Hill, Inc. New York, 1995) pp. 2625-2639)

Treatments for patients suffering from this disease include administration of analgesics for relief of bone pain, blood and platelet transfusions and, in some cases, splenectomy. Joint replacement is sometimes necessary for patients who experience bone erosion.

Enzyme replacement therapy with GCB has been used as a treatment for Gaucher disease. Current treatment of patients with Gaucher disease includes administration of a carbohydrate remodeled GCB derived from human placenta or Chinese hamster ovary (CHO) cells transfected with a GCB expression construct and known as alglucerase or imiglucerase, respectively. The treatment is extremely expensive in part because of the cost of removing sugars from GCB to expose the trimannosyl core of complex glycans in order to target the enzyme to mannose receptors on cells of reticuloendothelial origin. The scarcity of the human placental tissue (in the case of alglucerase), complex purification protocols, and the relatively large amounts of the carbohydrate remodeled GCB required all contribute to the cost of the treatment.

SUMMARY OF THE INVENTION

The invention is based, in part, on the discovery that by preventing removal of one or more mannose residues distal from the pentasaccharide core of a precursor oligosaccharide chain of a protein, e.g., a lysosomal storage enzyme, a high mannose protein such as high mannose glucocerebrosidase (hmGCB) can be obtained. These high mannose proteins can be used to target the protein to cells which express mannose receptors. Such cells can include cells of reticuloendothelial origin including macrophages, Kupffer cells and histiocytes. Thus, these high mannose proteins can be used, for example, to target delivery by receptor mediated endocytosis to lysosomes to treat various lysosomal storage diseases.

In particular, hmGCB has been found to efficiently target mannose receptors. Mannose receptors are present on macrophages and other cells, e.g., dendritic cells, cardiomyocytes and glial cells, and are instrumental in receptor-mediated endocytosis. The absence of GCB in patients with Gaucher disease leads to accumulation of glucocerebroside, primarily in cells of reticuloendothelial origin including macrophages, Kupffer cells and histiocytes. Because these cells express mannose receptors on their surface, hmGCB can be used to effectively target delivery of a corrective enzyme to the lysosomes through receptor-mediated

endocytosis, thereby treating Gaucher disease. Surprisingly, it was found that hmGCB uptake by macrophages was increased as compared to uptake of GCB secreted from cells.

Accordingly, in one aspect, the invention features a method of producing a preparation of high mannose glucocerebrosidase (hmGCB). The method includes:

providing a cell which is capable of expressing GCB; and allowing production of GCB having a precursor oligosaccharide under conditions which prevent the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB, to thereby produce an hmGCB preparation.

In a preferred embodiment, the GCB is human GCB. In a preferred embodiment, the cell is a human cell.

In a preferred embodiment, the removal of: one or more α 1.2 mannose residue(s) distal to the pentasaccharide core is prevented; an α 1.3 mannose residue distal to the pentasaccharide core is prevented; and/or an α 1.6 mannose residue distal to the pentasaccharide core is prevented. Preferably, the removal of one or more α 1.2 mannose residue(s) distal to the pentasaccharide core is prevented.

In a preferred embodiment, the method can include contacting the cell with a substance which prevents the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB, e.g., prevents removal of one or more α 1.2-mannose residue(s) distal to the pentasaccharide core, an α 1.3 mannose residue distal to the pentasaccharide core and/or an α 1.6 mannose residue distal to the pentasaccharide core. Preferably, the removal of one or more α 1.2 mannose(s) residue distal to the pentasaccharide core is prevented.

In a preferred embodiment, the method includes contacting the cell with a substance which prevents the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB, wherein the substance is a mannosidase inhibitor. The mannosidase inhibitor can be a class 1 processing mannosidase inhibitor, a class 2 processing mannosidase inhibitor or both. The class 1 processing mannosidase inhibitor can be one or more of: kifunensine, deoxymannojirimycin, or a similar inhibitor. Preferably, the class 1 processing mannosidase inhibitor is kifunensine. Useful class 2 processing mannosidase inhibitors can include one or more of: swainsonine, mannostatin, 6-deoxy-1,4-dideoxy-1,4-imino-D-mannitol (6-deoxy-DIM), and 6-deoxy-6-fluoro-1,4-dideoxy-1,4-imino-D-mannitol (6-deoxy-6-fluoro-DIM). Preferably, the class 2 processing mannosidase inhibitor is swainsonine.

In a preferred embodiment, a mannosidase inhibitor is present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml.

In a preferred embodiment, the method further includes contacting the cell with a class 1 processing mannosidase inhibitor and a class 2 processing mannosidase inhibitor. In a preferred embodiment, the class 1 processing mannosidase inhibitor is present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; the class 2 processing mannosidase inhibitor is present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; each of the class 1 processing and class 2 processing mannosidase inhibitors are present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; the total concentration of the class 1 processing and class 2 processing mannosidase inhibitors

present is between about 0.025 to 40.0 µg/ml, 0.05 to 20 µg/ml, 0.05 to 10 µg/ml, preferably between about 0.1 to 4.0 µg/ml.

In a preferred embodiment, the cell carries a mutation for, e.g., a knockout for, at least one Golgi processing mannosidase. The mutation can be one which reduces the expression of the gene, reduces protein or activity levels, or alters the distribution or other post translational modifications of the mannosidase, e.g., the processing of the carbohydrate chains. The mutation can be one which reduces the level of the Golgi processing mannosidase activity, e.g., one which reduces gene expression, e.g., a null mutation, e.g., a deletion, a frameshift or an insertion. In a preferred embodiment the mutation is a knockout, e.g., in the mannosidase gene. The mutation can affect the structure (and activity of the protein), and can, e.g., be a temperature sensitive mutation or a truncation. In a preferred embodiment, the cell carries a mutation, e.g., a knockout, for: a class 1 processing mannosidase; a class 2 processing mannosidase; a class 1 processing mannosidase and a class 2 processing mannosidase. In a preferred embodiment, the class 1 processing mannosidase is: Golgi mannosidase IA; Golgi mannosidase IB; Golgi mannosidase IC; or combinations thereof. In a preferred embodiment, the class 2 processing mannosidase is: Golgi mannosidase II.

In a preferred embodiment, the cell includes a nucleic acid sequence, such as an antisense molecule or ribozyme, which can bind to or inactivate a cellular mannosidase nucleic acid sequence, e.g., mRNA, and inhibit expression of the protein. In a preferred embodiment, the nucleic acid sequence is: a class 1 processing mannosidase antisense molecule; a class 2 processing mannosidase antisense molecule; both a class 1 processing mannosidase antisense molecule and a class 2 processing mannosidase antisense molecule. In a preferred embodiment, the class 1 processing mannosidase is: Golgi mannosidase IA; Golgi mannosidase IB; Golgi mannosidase IC; and combinations thereof. In a preferred embodiment, the class 2 processing mannosidase is: Golgi mannosidase II.

In a preferred embodiment, the cell includes a molecule, e.g., an exogenously supplied molecule, which binds and inhibits a mannosidase. The molecule can be, e.g., a single chain antibody, an intracellular protein or a competitive or non-competitive inhibitor.

In a preferred embodiment, the hmGCB molecule includes a carbohydrate chain having at least four mannose residues. For example, the molecule has at least one carbohydrate chain having five mannose residues, the hmGCB molecule has at least one carbohydrate chain having six mannose residues, the hmGCB molecule has at least one carbohydrate chain having seven mannose residues, the hmGCB molecule has at least one carbohydrate chain having eight mannose residues, the hmGCB molecule has at least one carbohydrate chain having nine mannose residues. Preferably, the hmGCB molecule has at least one carbohydrate chain having five, eight or nine mannose residues.

In a preferred embodiment, the hmGCB produced (either one or more hmGCB molecules or the preparation as a whole) has a ratio of mannose residues to GlcNAc residues which is greater than 3 mannose residues to 2 GlcNAc residues, preferably the ratio of mannose to GlcNAc is 4:2, 5:2, 6:2, 7:2, 8:2, 9:2, more preferably the ratio of mannose to GlcNAc is 5:2, 8:2 or 9:2.

In a preferred embodiment, the removal of one or more mannose residues distal to the pentasaccharide core is prevented on one, two, three or four of the carbohydrate chains of an hmGCB molecule.

In a preferred embodiment, at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or all of the hmGCB molecules of the preparation have at least one, and preferably two, three or four carbohydrate chains in which the removal of one or more mannose residues distal to the pentasaccharide core has been prevented.

In a preferred embodiment, the hmGCB preparation is a relatively heterogeneous preparation. Preferably, less than 80%, 70%, 60%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5% or 1% of the carbohydrate chains in the hmGCB preparation have the same number of mannose residues in addition to the pentasaccharide core. For example, the ratio of carbohydrate chains having the same number of mannose residues in addition to the pentasaccharide core to carbohydrate chains having a different number of mannose residues can be about: 60%:40%; 50%:50%; 40%:60%; 30%:70%; 25%:75%; 20%:80%; 15%:85%; 10%:90%; 5% or less:95% or more.

In a preferred embodiment, activity of Golgi mannosidase IA and/or IB and/or IC is inhibited and at least about 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or 100% of the carbohydrate chains in the hmGCB preparation have five or more mannose residues, e.g., five, six, seven, eight and/or nine mannose residues. In a preferred embodiment, activity of Golgi mannosidase I is inhibited and the ratio of carbohydrate chains having five or more mannose residues to carbohydrate chains having four or less mannose residues is about 60%:40%; 70%:30%; 75%:25%; 80%:20%; 85%:15%; 90%:10%; 95%:5%; 99%:1% or 100%:0%.

In a preferred embodiment, activity of Golgi mannosidase II is inhibited and at least about 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or 100% of the carbohydrate chains in the hmGCB preparation have five or more mannose residues, e.g., five, six, seven, eight and/or nine mannose residues. In a preferred embodiment, activity of Golgi mannosidase II is inhibited and the ratio of carbohydrate chains having five or more mannose residues to carbohydrate chains having four or less mannose residues is about 60%:40%; 70%:30%; 75%:25%; 80%:20%; 85%:15%; 90%:10%; 95%:5%; 99%:1% or 100%:0%.

In a preferred embodiment, the cell includes an exogenous nucleic acid sequence which includes a GCB coding region. In a preferred embodiment, the cell further includes a regulatory sequence, an endogenous or exogenous regulatory sequence, which functions to regulate expression of the exogenous GCB coding region.

In a preferred embodiment, the cell includes an exogenous regulatory sequence which functions to regulate expression of an endogenous GCB coding sequence, e.g., the regulatory sequence is integrated into the genome of the cell such that it regulates expression of an endogenous GCB coding sequence.

In a preferred embodiment, the regulatory sequence includes one or more of: a promoter, an enhancer, an upstream activating sequence (UAS), a scaffold-attachment region or a transcription factor-binding site. In a preferred embodiment, the regulatory sequence includes: a regulatory sequence from a metallothionein-I gene, e.g., a mouse metallothionein-I gene, a regulatory sequence from an SV-40 gene, a regulatory sequence from a cytomegalovirus gene, a regulatory sequence from a collagen gene, a regulatory sequence from an actin gene, a regulatory sequence from an immunoglobulin gene, a regulatory sequence from the HMG-CoA reductase gene, or a regulatory sequence from the EF-1 α gene.

In a preferred embodiment, the cell is: a eukaryotic cell. In a preferred embodiment, the cell is of fungal, plant or

animal origin, e.g., vertebrate origin. In a preferred embodiment, the cell is: a mammalian cell, e.g., a primary or secondary mammalian cell, e.g., a fibroblast, a hematopoietic stem cell, a myoblast, a keratinocyte, an epithelial cell, an endothelial cell, a glial cell, a neural cell, a cell comprising a formed element of the blood, a muscle cell and precursors of these somatic cells; a transformed or immortalized cell line. Preferably, the cell is a human cell. Examples of immortalized human cell lines useful in the present method include, but are not limited to: a Bowes Melanoma cell (ATCC Accession No. CRL 9607), a Daudi cell (ATCC Accession No. CCL 213), a HeLa cell and a derivative of a HeLa cell (ATCC Accession Nos. CCL2 CCL2.1 and CCL 2.2), a HL-60 cell (ATCC Accession No. CCL 240), an HT-1080 cell (ATCC Accession No. CCL 121), a Jurkat cell (ATCC Accession No. TIB 152), a KB carcinoma cell (ATCC Accession No. CCL 17), a K-562 leukemia cell (ATCC Accession No. CCL 243), a MCF-7 breast cancer cell (ATCC Accession No. BTH 22), a MOLT-4 cell (ATCC Accession No. 1582), a Namalwa cell (ATCC Accession No. CRL 1432), a Raji cell (ATCC Accession No. CCL 86), a RPMI 8226 cell (ATCC Accession No. CCL 155), a U-937 cell (ATCC Accession No. 1593), WI-28VA13 sub line 2R4 cells (ATCC Accession No. CCL 155), a CCRF-CEM cell (ATCC Accession No. CCL 119) and a 2780AD ovarian carcinoma cell (Van Der Blick et al., Cancer Res. 48:5927-5932, 1988), as well as hetero-hybridoma cells produced by fusion of human cells and cells of another species. In another embodiment, the immortalized cell line can be cell line other than a human cell line, e.g., a CHO cell line, a COS cell line. In another embodiment, the cell can be a from a clonal cell strain or clonal cell line.

In a preferred embodiment, a population of cells which are capable of expressing hmGCB is provided, and at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or all of the cells produce hmGCB molecules with at least one carbohydrate chain, and preferably two, three, or four carbohydrate chains, having the specified number of mannose residues.

In a preferred embodiment, the cell is cultured in culture medium which includes at least one mannosidase inhibitor. In a preferred embodiment, the method further includes obtaining the hmGCB from the medium in which the cell is cultured.

In another aspect, the invention features a method of producing a preparation of hmGCB. The method includes: providing a cell which is capable of expressing GCB; and allowing production of GCB having a precursor oligosaccharide under conditions which inhibit class 1 processing mannosidase activity and class 2 processing mannosidase activity such that the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB is prevented, to thereby produce an hmGCB preparation.

In a preferred embodiment, the GCB is human GCB. In a preferred embodiment, the cell is a human cell.

In a preferred embodiment, the removal of one or more α 1,2 mannose residue(s) distal to the pentasaccharide core is prevented; an α 1,3 mannose residue distal to the pentasaccharide core is prevented; and/or an α 1,6 mannose residue distal to the pentasaccharide core is prevented. Preferably, the removal of one or more α 1,2 mannose residue(s) distal to the pentasaccharide core is prevented.

In a preferred embodiment, the method can include: contacting the cell with a substance which inhibits a class 1 processing mannosidase activity and a substance which inhibits a class 2 processing mannosidase activity thereby

preventing the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB. In a preferred embodiment, the substances prevent removal of one or more α 1,2 mannose residue distal to the pentasaccharide core.

In a preferred embodiment, the method includes contacting the cell with a substance which inhibits a class 1 processing mannosidase activity and a substance which inhibits a class 2 processing mannosidase activity, wherein the substances are a class 1 processing mannosidase inhibitor and a class 2 processing mannosidase inhibitor. In a preferred embodiment, the class 1 processing mannosidase inhibitor can be one or more of: kifunensine, deoxymannojirimycin, or a similar inhibitor. Preferably, the class 1 processing mannosidase inhibitor is kifunensine. In a preferred embodiment, the class 2 processing mannosidase inhibitor can be one or more of: swainsonine, mannosatin, 6-deoxy-DIM, and 6-deoxy-6-fluoro-DIM. Preferably, the class 2 processing mannosidase inhibitor is swainsonine.

In a preferred embodiment, a class 1 processing mannosidase inhibitor is present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; a class 2 processing mannosidase inhibitor is present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; each of the class 1 processing and class 2 processing mannosidase inhibitors are present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; the total concentration of the class 1 processing and class 2 processing mannosidase inhibitors present is between about 0.025 to 40.0 μ g/ml, 0.05 to 20 μ g/ml, 0.05 to 10 μ g/ml, preferably between about 0.1 to 4.0 μ g/ml.

In a preferred embodiment, the cell carries a mutation for, e.g., a knockout for, a class 1 mannosidase and a class 2 mannosidase. The mutation can be one which reduces the expression of the gene, reduces protein or activity levels, or alters the distribution or other post translational modifications of the mannosidase, e.g., the processing of the carbohydrate chains. The mutation can be one which reduces the level of a class 1 processing mannosidase and/or a class 2 processing mannosidase activity, e.g., one which reduces gene expression, e.g., a null mutation, e.g., a deletion, a frameshift, or an insertion. In a preferred embodiment, the mutation is a knockout in the mannosidase gene. The mutation can affect the structure (and activity of the protein), and can, e.g., be a temperature sensitive mutant. In a preferred embodiment, the class 1 processing mannosidase is: Golgi mannosidase IA; Golgi mannosidase IB; Golgi mannosidase IC; combinations thereof. In a preferred embodiment, the class 2 processing mannosidase is: Golgi mannosidase II.

In a preferred embodiment, the cell includes both a class 1 processing mannosidase antisense molecule and a class 2 processing mannosidase antisense molecule. In a preferred embodiment, the class 1 processing mannosidase is: Golgi mannosidase IA; Golgi mannosidase IB; Golgi mannosidase IC; combinations thereof. In a preferred embodiment, the class 2 processing mannosidase is: Golgi mannosidase II.

In a preferred embodiment, the cell includes a molecule, e.g., an exogenously supplied molecule, which binds and inhibits a mannosidase. The molecule can be, e.g., a single chain antibody, an intracellular protein or a competitive or non-competitive inhibitor.

In a preferred embodiment, the class 1 processing mannosidase activity and the class 2 mannosidase activity can be

inhibited by different mechanisms. For example, a class 1 processing mannosidase activity can be inhibited by contacting the cell with a substrate which inhibits a class 1 processing mannosidase, e.g., a class 1 mannosidase inhibitor, and the class 2 processing mannosidase can be inhibited by using a cell which is a knockout of a class 2 mannosidase and/or includes a class 2 mannosidase antisense molecule. In another preferred embodiment, a class 2 processing mannosidase activity can be inhibited by contacting the cell with a substrate which inhibits a class 2 processing mannosidase, e.g., a class 2 mannosidase inhibitor, and the class 1 processing mannosidase can be inhibited by using a cell which is a knockout of a class 1 mannosidase and/or includes a class 1 mannosidase antisense molecule.

In a preferred embodiment, the hmGCB molecule includes a carbohydrate chain having at least four mannose residues. For example, the hmGCB molecule has at least one carbohydrate chain having five mannose residues, the hmGCB molecule has at least one carbohydrate chain having six mannose residues, the hmGCB molecule has at least one carbohydrate chain having seven mannose residues, the hmGCB molecule has at least one carbohydrate chain having eight mannose residues, the hmGCB molecule has at least one carbohydrate chain having nine mannose residues. Preferably, the hmGCB molecule has at least one carbohydrate chain having five, eight or nine mannose residues.

In a preferred embodiment, the hmGCB produced (either one or more hmGCB molecules or the preparation as a whole) has a ratio of mannose residues to GlcNAc residues which is greater than 3 mannose residues to 2 GlcNAc residues, preferably the ratio of mannose to GlcNAc is 4:2, 5:2, 6:2, 7:2, 8:2, 9:2, more preferably the ratio of mannose to GlcNAc is 8:2 or 9:2.

In a preferred embodiment, the removal of one or more mannose residues distal to the pentasaccharide core is prevented on one, two, three or four of the carbohydrate chains of the hmGCB molecule.

In a preferred embodiment, at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or all of the hmGCB molecules of the preparation have at least one, and preferably two, three or four carbohydrate chains in which the removal of one or more mannose residues distal to the pentasaccharide core has been prevented.

In a preferred embodiment, the hmGCB preparation is a relatively heterogeneous preparation. Preferably, less than 80%, 70%, 60%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5% or 1% of the carbohydrate chains in the hmGCB preparation have the same number of mannose residues in addition to the pentasaccharide core. For example, the ratio of carbohydrate chains having the same number of mannose residues in addition to the pentasaccharide core to carbohydrate chains having a different number of mannose residues can be about: 60%:40%; 50%:50%; 40%:60%; 30%:70%; 25%:75%; 20%:80%; 15%:85%; 10%:90%; 5% or less:95% or more.

In a preferred embodiment, activity of a class 1 processing mannosidase, e.g., Golgi mannosidase IA and/or Golgi mannosidase IB and/or Golgi mannosidase IC, and activity of a class 2 processing mannosidase, e.g., Golgi mannosidase II, are inhibited and at least about 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or 100% of the carbohydrate chains in the hmGCB preparation have five or more mannose residues, e.g., five, six, seven, eight and/or nine mannose residues. In a preferred embodiment, activity of a class 1 processing mannosidase, e.g., Golgi mannosidase IA and/or Golgi mannosidase IB and/or Golgi mannosidase IC, and activity of a class 2 processing mannosidase, e.g., Golgi

mannosidase II, are inhibited and the ratio of carbohydrate chains having five or more mannose residues to carbohydrate chains having four or less mannose residues, respectively, is about 60%:40%; 70%:30%; 75%:25%; 80%:20%; 85%:15%; 90%:10%; 95%:5%; 99%:1%; or 100%:0%.

In a preferred embodiment, the cell includes an exogenous nucleic acid sequence which includes a GCB coding region. In a preferred embodiment, the cell further includes a regulatory sequence, an endogenous or exogenous regulatory sequence, which functions to regulate expression of the exogenous GCB coding region.

In a preferred embodiment, the cell includes an exogenous regulatory sequence which functions to regulate expression of an endogenous GCB coding sequence, e.g., the regulatory sequence is integrated into the genome of the cell such that it regulates expression of an endogenous GCB coding sequence.

In a preferred embodiment, the regulatory sequence includes one or more of: a promoter, an enhancer, an upstream activating sequence (UAS), a scaffold-attachment region or a transcription factor-binding site. In a preferred embodiment, the regulatory sequence includes: a regulatory sequence from a metallothionein-I gene, e.g., a mouse metallothionein-I gene, a regulatory sequence from an SV-40 gene, a regulatory sequence from a cytomegalovirus gene, a regulatory sequence from a collagen gene, a regulatory sequence from an actin gene, a regulatory sequence from an immunoglobulin gene, a regulatory sequence from the HMG-CoA reductase gene, or a regulatory sequence from the EF-1 α gene.

In a preferred embodiment, the cell is: a eukaryotic cell. In a preferred embodiment, the cell is of fungal, plant or animal origin, e.g., vertebrate origin. In a preferred embodiment, the cell is: a mammalian cell, e.g., a primary or secondary mammalian cell, e.g., a fibroblast, a hematopoietic stem cell, a myoblast, a keratinocyte, an epithelial cell, an endothelial cell, a glial cell, a neural cell, a cell comprising a formed element of the blood, a muscle cell and precursors of these somatic cells; a transformed or immortalized cell line. Preferably, the cell is a human cell. Examples of immortalized human cell lines useful in the present method include, but are not limited to: a Bowes Melanoma cell (ATCC Accession No. CRL 9607), a Daudi cell (ATCC Accession No. CCL 213), a HeLa cell and a derivative of a HeLa cell (ATCC Accession Nos. CCL2 CCL2.1 and CCL 2.2), a HI-60 cell (ATCC Accession No. CCL 240), an HT-1080 cell (ATCC Accession No. CCL 121), a Jurkat cell (ATCC Accession No. TIB 152), a KB carcinoma cell (ATCC Accession No. CCL 17), a K-562 leukemia cell (ATCC Accession No. CCL 243), a MCF-7 breast cancer cell (ATCC Accession No. BTH 22), a MOLT-4 cell (ATCC Accession No. 1582), a Namalwa cell (ATCC Accession No. CRL 1432), a Raji cell (ATCC Accession No. CCL 86), a RPMI 8226 cell (ATCC Accession No. CCL 155), a U-937 cell (ATCC Accession No. 1593), WI-28VA13 sub line 2R4 cells (ATCC Accession No. CCL 155), a CCRF-CEM cell (ATCC Accession No. CCL 119) and a 2780AD ovarian carcinoma cell (Van Der Blick et al., Cancer Res. 48:5927-5932, 1988), as well as heterohybridoma cells produced by fusion of human cells and cells of another species. In another embodiment, the immortalized cell line can be cell line other than a human cell line, e.g., a CHO cell line, a COS cell line. In another embodiment, the cell can be from a clonal cell strain or clonal cell line.

In a preferred embodiment, a population of cells which are capable of expressing hmGCB is provided, and at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%.

98%, 99% or all of the cells produce hmGCB with at least one carbohydrate chain, preferably two, three, or four carbohydrate chains, having the specified number of mannose residues.

In a preferred embodiment, the cell is cultured in a culture medium which includes at least one class 1 processing mannosidase inhibitor and at least one class 2 processing mannosidase inhibitor. In a preferred embodiment, the method further includes obtaining the hmGCB from the medium in which the cell is cultured.

In another aspect, the invention features a method of producing a preparation of hmGCB. The method includes:

providing a cell into which a nucleic acid sequence comprising an exogenous regulatory sequence has been introduced such that the regulatory sequence regulates the expression of an endogenous GCB coding region; and

allowing production of GCB having a precursor oligosaccharide under conditions which prevent the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB, to thereby produce an hmGCB preparation.

In a preferred embodiment, the GCB is human GCB.

In a preferred embodiment, the removal of: one or more α 1,2 mannose residue(s) distal to the pentasaccharide core is prevented; an α 1,3 mannose residue distal to the pentasaccharide core is prevented; and/or an α 1,6 mannose residue distal to the pentasaccharide core is prevented. Preferably, the removal of one or more α 1,2 mannose residue(s) distal to the pentasaccharide core is prevented.

In a preferred embodiment, the method can include contacting the cell with a substance which prevents the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB, e.g., prevents removal of one or more α 1,2 mannose residue(s) distal to the pentasaccharide core, an α 1,3 mannose residue distal to the pentasaccharide core and/or an α 1,6 mannose residue distal to the pentasaccharide core. Preferably, the removal of one or more α 1,2 mannose(s) residue distal to the pentasaccharide core is prevented.

In a preferred embodiment, the method includes contacting the cell with a substance which prevents the removal of at least one mannose residue distal to the pentasaccharide core of the precursor oligosaccharide of GCB, and the substance is a mannosidase inhibitor. The mannosidase inhibitor can be a class 1 processing mannosidase inhibitor, a class 2 processing mannosidase inhibitor or both. The class 1 processing mannosidase inhibitor can be one or more of: kifunensine, deoxymannojirimycin, or a similar inhibitor. Preferably, the class 1 processing mannosidase inhibitor is kifunensine. Useful class 2 processing mannosidase inhibitors can include one or more of: swainsonine, mannosatin, 6-deoxy-DJM, 6-deoxy-6-fluoro-DJM. Preferably, the class 2 processing mannosidase inhibitor is swainsonine.

In a preferred embodiment, a mannosidase inhibitor is present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml.

In a preferred embodiment, the method further includes contacting the cell with a class 1 processing mannosidase inhibitor and a class 2 processing mannosidase inhibitor. In a preferred embodiment, a class 1 processing mannosidase inhibitor is present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; a class 2 processing mannosidase inhibitor is present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; each of the class

1 processing and class 2 processing mannosidase inhibitors are present at a concentration between about 0.025 to 20.0 μ g/ml, 0.05 to 10 μ g/ml, 0.05 to 5 μ g/ml, preferably between about 0.1 to 2.0 μ g/ml; the total concentration of the class 1 processing and class 2 processing mannosidase inhibitors present is between about 0.025 to 40.0 μ g/ml, 0.05 to 20 μ g/ml, 0.05 to 10 μ g/ml, preferably between about 0.1 to 4.0 μ g/ml.

In a preferred embodiment, the cell carries a mutation for, e.g., a knockout for, at least one mannosidase. The mutation can be one which reduces the expression of the gene, reduces protein or activity levels, or alters the distribution or other post translational modifications of the mannosidase, e.g., the processing of the carbohydrate chains. The mutation can be one which reduces the level of the Golgi processing mannosidase activity, e.g., one which reduces gene expression, e.g., a null mutation, e.g., a deletion, a frameshift or an insertion. In a preferred embodiment the mutation is a knockout, e.g., in the mannosidase gene. The mutation can affect the structure (and activity of the protein), and can, e.g., be a temperature sensitive mutation or a truncation. In a preferred embodiment, the cell carries a mutation, e.g., a knockout, for: a class 1 processing mannosidase; a class 2 processing mannosidase; a mutant, e.g., a knockout, for a class 1 processing mannosidase and a class 2 processing mannosidase. In a preferred embodiment, the class 1 processing mannosidase is: Golgi mannosidase IA; Golgi mannosidase IB; Golgi mannosidase IC; or combinations thereof. In a preferred embodiment, the class 2 processing mannosidase is: Golgi mannosidase II.

In a preferred embodiment, the cell includes a nucleic acid sequence, such as an antisense molecule or ribozyme, which can bind to or inactivate a cellular mannosidase nucleic acid sequence, e.g., mRNA, and inhibit expression of the protein. In a preferred embodiment, the nucleic acid sequence is: a class 1 processing mannosidase antisense molecule; a class 2 processing mannosidase antisense molecule; both a class 1 processing mannosidase antisense molecule and a class 2 processing mannosidase antisense molecule. In a preferred embodiment, the class 1 processing mannosidase is: Golgi mannosidase IA; Golgi mannosidase IB; Golgi mannosidase IC; combinations thereof. In a preferred embodiment, the class 2 processing mannosidase is: Golgi mannosidase II.

In a preferred embodiment, the cell includes a molecule, e.g., an exogenously supplied molecule, which binds and inhibits a mannosidase. The molecule can be, e.g., a single chain antibody, an intracellular protein or a competitive or non-competitive inhibitor.

In a preferred embodiment, the hmGCB molecule includes a carbohydrate chain having at least four mannose residues. For example, the hmGCB molecule has at least one carbohydrate chain having five mannose residues, the hmGCB molecule has at least one carbohydrate chain having six mannose residues, the hmGCB molecule has at least one carbohydrate chain having seven mannose residues, the hmGCB molecule has at least one carbohydrate chain having eight mannose residues, the hmGCB molecule has at least one carbohydrate chain having nine mannose residues. Preferably, the hmGCB molecule has at least one carbohydrate chain having five, eight or nine mannose residues.

In a preferred embodiment, the hmGCB produced (either one or more hmGCB molecules or the preparation as a whole) has a ratio of mannose residues to GlcNAc residues which is greater than 3 mannose residues to 2 GlcNAc residues, preferably the ratio of mannose to GlcNAc is 4:2, 5:2, 6:2, 7:2, 8:2, 9:2, more preferably the ratio of mannose to GlcNAc is 5:2, 8:2 or 9:2.

In a preferred embodiment, the removal of one or more mannose residues distal to the pentasaccharide core is prevented on one, two, three or four of the carbohydrate chains of the hmGCB molecule.

In a preferred embodiment, at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or all of the hmGCB molecules of the preparation have at least one, and preferably two, three or four carbohydrate chains in which the removal of one or more mannose residues distal to the pentasaccharide core has been prevented.

In a preferred embodiment, the hmGCB preparation is a relatively heterogeneous preparation. Preferably, less than 80%, 70%, 60%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5% or 1% of the carbohydrate chains in the hmGCB preparation have the same number of mannose residues in addition to the pentasaccharide core. For example, the ratio of carbohydrate chains having the same number of mannose residues in addition to the pentasaccharide core to carbohydrate chains having a different number of mannose residues can be about: 60%:40%; 50%:50%; 40%:60%; 30%:70%; 25%:75%; 20%:80%; 15%:85%; 10%:90%; 5% or less:95% or more.

In a preferred embodiment, activity of Golgi mannosidase IA and/or IB and/or IC is inhibited and at least about 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or 100% of the carbohydrate chains in the hmGCB preparation have five or more mannose residues, e.g., five, six, seven, eight, and/or nine mannose residues. In a preferred embodiment, activity of Golgi mannosidase I is inhibited and the ratio of carbohydrate chains having five or more mannose residues to carbohydrate chains having four or less mannose residues is about 60%:40%; 70%:30%; 75%:25%; 80%:20%; 85%:15%; 90%:10%; 95%:5%; 99%:1%; or 100%:0%.

In a preferred embodiment, activity of Golgi mannosidase II is inhibited and at least about 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or 100% of the carbohydrate chains in the hmGCB preparation have five or more mannose residues. In a preferred embodiment, activity of Golgi mannosidase II is inhibited and the ratio of carbohydrate chains having five or more mannose residues to carbohydrate chains having four or less mannose residues is about 60%:40%; 70%:30%; 75%:25%; 80%:20%; 85%:15%; 90%:10%; 95%:5%; 99%:1%; or 100%:0%.

In a preferred embodiment, the regulatory sequence includes one or more of: a promoter, an enhancer, an upstream activating sequence (UAS), a scaffold-attachment region or a transcription factor-binding site. In a preferred embodiment, the regulatory sequence includes: a regulatory sequence from a metallothionein-I gene, e.g., a mouse metallothionein-I gene, a regulatory sequence from an SV-40 gene, a regulatory sequence from a cytomegalovirus gene, a regulatory sequence from an actin gene, a regulatory sequence from an immunoglobulin gene, a regulatory sequence from the HMG-CoA reductase gene, or a regulatory sequence from the EF-1 α gene.

In a preferred embodiment, the cell is: a eukaryotic cell. In a preferred embodiment, the cell is of fungal, plant or animal origin, e.g., vertebrate origin. In a preferred embodiment, the cell is: a mammalian cell, e.g., a primary or secondary mammalian cell, e.g., a fibroblast, a hematopoietic stem cell, a myoblast, a keratinocyte, an epithelial cell, an endothelial cell, a glial cell, a neural cell, a cell comprising a formed element of the blood, a muscle cell and precursors of these somatic cells; a transformed or immortalized cell line. Preferably, the cell is a human cell. Examples of immortalized human cell lines useful in the

present method include, but are not limited to: a Bowes Melanoma cell (ATCC Accession No. CRL 9607), a Daudi cell (ATCC Accession No. CCL 213), a HeLa cell and a derivative of a HeLa cell (ATCC Accession Nos. CCL2, CCL2.1 and CCL 2.2), a HL-60 cell (ATCC Accession No. CCL 240), an HT-1080 cell (ATCC Accession No. CCL 121), a Jurkat cell (ATCC Accession No. TIB 152), a KB carcinoma cell (ATCC Accession No. CCL 17), a K-562 leukemia cell (ATCC Accession No. CCL 243), a MCF-7 breast cancer cell (ATCC Accession No. BTH 22), a MOLT-4 cell (ATCC Accession No. 1582), a Namalwa cell (ATCC Accession No. CRL 1432), a Raji cell (ATCC Accession No. CCL 86), a RPMI 8226 cell (ATCC Accession No. CCL 155), a U-937 cell (ATCC Accession No. 1593), WI-28VA13 sub line 2R4 cells (ATCC Accession No. CCL 155), a CCRF-CEM cell (ATCC Accession No. CCL 119) and a 2780AD ovarian carcinoma cell (Van Der Blick et al., Cancer Res. 48:5927-5932, 1988), as well as heterohybridoma cells produced by fusion of human cells and cells of another species. In another embodiment, the immortalized cell line can be cell line other than a human cell line, e.g., a CHO cell line, a COS cell line. In another embodiment, the cell can be from a clonal cell strain or clonal cell line.

In a preferred embodiment, a population of cells which are capable of expressing hmGCB is provided, and at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or all of the cells produce hmGCB with at least one carbohydrate chain, preferably two, three, or four carbohydrate chains, having the specified number of mannose residues.

In a preferred embodiment, the cell is cultured in culture medium which includes at least one mannosidase inhibitor. In a preferred embodiment, the method further includes obtaining the hmGCB from the medium in which the cell is cultured.

In another aspect, the invention features an hmGCB molecule, e.g., an hmGCB molecule described herein, e.g., a human hmGCB, produced by any of the methods described herein. Preferably, the hmGCB molecule includes at least one carbohydrate chain, preferably two, three, or four carbohydrate chains, having at least four mannose residues of a precursor oligosaccharide chain.

In another aspect, the invention features an hmGCB preparation which includes a portion of hmGCB molecules which include at least one carbohydrate chain, preferably two, three, or four carbohydrate chains, having at least four mannose residues of a precursor oligosaccharide chain. Preferably, the hmGCB preparation is produced by any of the methods described herein.

In a preferred embodiment, the hmGCB is human hmGCB.

In a preferred embodiment, the hmGCB molecule can have: at least one carbohydrate chain having five mannose residues; at least one carbohydrate chain having six mannose residues; at least one carbohydrate chain having seven mannose residues; at least one carbohydrate chain having eight mannose residues; at least one carbohydrate chain having nine mannose residues.

In a preferred embodiment, the hmGCB produced (either one or more hmGCB molecules or the preparation as a whole) has at least one carbohydrate chain having a ratio of mannose residues to GlcNAc residues which is greater than 3 mannose residues to 2 GlcNAc residues, preferably the ratio of mannose to GlcNAc is 4:2, 5:2, 6:2, 7:2, 8:2, 9:2, more preferably the ratio of mannose to GlcNAc is 5:2, 8:2 or 9:2.

In a preferred embodiment, at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or all of the hmGCB of the preparation have at least one, preferably, two, three or four carbohydrate chains in which the removal of one or more mannose residues distal to the pentasaccharide core has been prevented.

In another aspect, the invention features a cell having at least one mannosidase activity inhibited and which includes a nucleic acid sequence comprising an exogenous regulatory sequence which has been introduced such that the regulatory sequence regulates the expression of an endogenous GCB coding region, wherein the cell produces GCB in which the removal of at least one mannose residue distal to the pentasaccharide core of a precursor oligosaccharide of GCB is prevented.

In a preferred embodiment, the cell produces an hmGCB preparation, e.g., a human hmGCB preparation, in which the removal of: one or more α 1,2 mannose residue(s) distal to the pentasaccharide core is prevented; an α 1,3 mannose residue distal to the pentasaccharide core is prevented; and/or an α 1,6 mannose residue distal to the pentasaccharide core is prevented. Preferably, the removal of one or more α 1,2 mannose residue(s) distal to the pentasaccharide core is prevented.

In a preferred embodiment, at least one mannosidase activity in the cell has been inhibited by contacting the cell with a substance which inhibits a mannosidase. In a preferred embodiment, the substance is a mannosidase inhibitor. The mannosidase inhibitor can be a class 1 processing mannosidase inhibitor, a class 2 processing mannosidase inhibitor or both. In a preferred embodiment, the class 1 processing mannosidase inhibitor can be one or more of: kifunensine and deoxymannojirimycin. Preferably, the class 1 processing mannosidase inhibitor is kifunensine. In a preferred embodiment, the class 2 processing mannosidase inhibitor can be one or more of: swainsonine, mannosatin, 6-deoxy-DIM, and 6-deoxy-6-fluoro-DIM. Preferably, the class 2 processing mannosidase inhibitor is swainsonine.

In a preferred embodiment, the cell carries a mutation for, e.g., a knockout for, at least one Golgi processing mannosidase. The mutation can be one which reduces the expression of the gene, reduces protein or activity levels, or alters the distribution or other post translational modifications of the mannosidase, e.g., the processing of a carbohydrate chain. The mutant can be one which reduces the level of Golgi processing mannosidase activity, e.g., one which reduces gene expression, e.g., a null mutation, e.g., a deletion, a frameshift, or an insertion. In a preferred embodiment, the mutation is a knockout in the mannosidase gene. The mutation can affect the structure (and activity of the protein), and can, e.g., be a temperature sensitive mutation. In a preferred embodiment, the cell is a mutant, e.g., a knockout, for: a class 1 processing mannosidase; a class 2 processing mannosidase; a class 1 processing mannosidase and a class 2 processing mannosidase. In a preferred embodiment, the class 1 processing mannosidase is: Golgi mannosidase IA; Golgi mannosidase IB; Golgi mannosidase IC; combinations thereof. In a preferred embodiment, the class 2 processing mannosidase is: Golgi mannosidase II.

In a preferred embodiment, the cell further includes a nucleic acid sequence, such as an antisense molecule or ribozyme, which can bind to or inactivate a cellular mannosidase nucleic acid sequence, e.g., mRNA, and inhibit expression of the protein. In a preferred embodiment, the nucleic acid sequence is: a class 1 processing mannosidase antisense molecule; a class 2 processing mannosidase antisense molecule; both a class 1 processing mannosidase

antisense molecule and a class 2 processing mannosidase antisense molecule. In a preferred embodiment, the class 1 processing mannosidase is: Golgi mannosidase IA; Golgi mannosidase IB; Golgi mannosidase IC; combinations thereof. In a preferred embodiment, the class 2 processing mannosidase is: Golgi mannosidase II.

In a preferred embodiment, the cell includes a molecule, e.g., an exogenously supplied molecule, which binds and inhibits a mannosidase. The molecule can be, e.g., a single chain antibody, an intracellular protein or a competitive or non-competitive inhibitor.

In a preferred embodiment, the hmGCB molecule produced by the cell has a ratio of mannose residues to GlcNAc residues which is greater than 3 mannose residues to 2 GlcNAc residues, preferably the ratio of mannose to GlcNAc is 4:2, 5:2, 6:2, 7:2, 8:2, 9:2, more preferably the ratio of mannose to GlcNAc is 5:2, 8:2 or 9:2.

In a preferred embodiment, the cell is unable to remove of one or more mannose residues distal to the pentasaccharide core on one, two, three or four of the carbohydrate chains of hmGCB.

In a preferred embodiment, at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or all of the hmGCB molecules produced by the cell have at least one, preferably, two, three or four carbohydrate chains in which the removal of one or more mannose residues distal to the pentasaccharide core has been prevented.

In a preferred embodiment, the regulatory sequence includes one or more of: a promoter, an enhancer, an upstream activating sequence (UAS), a scaffold-attachment region or a transcription factor-binding site. In a preferred embodiment, the regulatory sequence includes: a regulatory sequence from a metallothionein-I gene, e.g., a mouse metallothionein-I gene, a regulatory sequence from an SV-40 gene, a regulatory sequence from a cytomegalovirus gene, a regulatory sequence from a collagen gene, a regulatory sequence from an actin gene, a regulatory sequence from an immunoglobulin gene, a regulatory sequence from the HMG-CoA reductase gene, or a regulatory sequence from the EF-1 α gene.

In a preferred embodiment, the cell is: a eukaryotic cell. In a preferred embodiment, the cell is of fungal, plant or animal origin, e.g., vertebrate origin. In a preferred embodiment, the cell is: a mammalian cell, e.g., a primary or secondary mammalian cell, e.g., a fibroblast, a hematopoietic stem cell, a myoblast, a keratinocyte, an epithelial cell, an endothelial cell, a glial cell, a neural cell, a cell comprising a formed element of the blood, a muscle cell and precursors of these somatic cells; a transformed or immortalized cell line. Preferably, the cell is a human cell. Examples of immortalized human cell lines useful in the present method include, but are not limited to: a Bowes Melanoma cell (ATCC Accession No. CRL 9607), a Daudi cell (ATCC Accession No. CCL 213), a HeLa cell and a derivative of a HeLa cell (ATCC Accession Nos. CCL2, CCL2.1, and CCL 2.2), a HL-60 cell (ATCC Accession No. CCL 240), an HT-1080 cell (ATCC Accession No. CCL 121), a Jurkat cell (ATCC Accession No. TIB 152), a KB carcinoma cell (ATCC Accession No. CCL 17), a K-562 leukemia cell (ATCC Accession No. CCL 243), a MCF-7 breast cancer cell (ATCC Accession No. BTH 22), a MOLT-4 cell (ATCC Accession No. 1582), a Namalwa cell (ATCC Accession No. CRL 1432), a Raji cell (ATCC Accession No. CCL 86), a RPMI 8226 cell (ATCC Accession No. CCL 155), a U-937 cell (ATCC Accession No. 1593), WI-28VA13 sub line 2R4 cells (ATCC Accession No. CLL 155), a CCRF-CEM cell (ATCC Accession No. CCL

119) and a 2780AD ovarian carcinoma cell (Van Der Blick et al., Cancer Res. 48:5927-5932, 1988), as well as hetero-hybridoma cells produced by fusion of human cells and cells of another species. In another embodiment, the immortalized cell line can be cell line other than a human cell line, e.g., a CHO cell line, a COS cell line. In another embodiment, the cell can be from a clonal cell strain or clonal cell line.

In another aspect, the invention features a pharmaceutical composition which includes an hmGCB molecule, e.g., a human hmGCB, which includes at least one carbohydrate chain, preferably two, three, or four carbohydrate chains, having at least four mannose residues of a precursor oligosaccharide chain, in an amount suitable for treating Gaucher disease.

In a preferred embodiment, the pharmaceutical composition further includes a pharmaceutically acceptable carrier or diluent.

Another aspect of the invention features a method of treating a subject having Gaucher disease. The method includes administering to a subject having Gaucher disease an hmGCB preparation, e.g., a human hmGCB preparation, which includes at least one carbohydrate chain, preferably two, three, or four carbohydrate chains, having at least four mannose residues of a precursor oligosaccharide chain, in an amount suitable for treating Gaucher disease.

In another aspect, the invention features a method of purifying hmGCB from a sample. The method includes: providing a harvested hmGCB product; and subjecting the hmGCB product to hydrophobic charge induction chromatography (HCIC) and/or hydrophobic interaction chromatography (HIC), thereby obtaining purified hmGCB.

In a preferred embodiment, chromatography material MEP HYPERCEL® is used for HCIC. In another preferred embodiment, chromatography material MACROPREP METHYL® is used for HIC.

In another preferred embodiment, the method further includes subjecting the hmGCB product to ion exchange chromatography. The hmGCB product can be subjected to HCIC and/or HIC prior to ion exchange chromatography or the hmGCB product can be subjected to ion exchange chromatography prior to HCIC and/or HIC. Preferably, the hmGCB product is subjected to more than one ion exchange chromatography step. The ion exchange chromatography can be: anion exchange chromatography, cation exchange chromatography or both.

In a preferred embodiment, anion exchange chromatography is performed using one or more of the following chromatography materials: Q SEPHAROSE FAST FLOW®, MACROPREP HIGH Q SUPPORT®, DEAE SEPHAROSE FAST FLOW®, AND MACRO-PREP DEAE®. In a preferred embodiment, cation exchange chromatography is performed using one or more of: SP Sepharose Fast Flow®, Source 30S®, CM Sepharose Fast Flow®, Macro-Prep CM Support®, and Macro-Prep High S Support®.

In a preferred embodiment, the method further includes subjecting the hmGCB product to size exclusion chromatography. Preferably, the size exclusion chromatography is performed using one or more of the following chromatography materials: SUPERDEX 200®, SEPHACRYL S-200 HR® AND BIO-GEL A 1.5M®.

In another aspect, the invention features a method of purifying hmGCB. The method includes: providing a harvested hmGCB product; subjecting the hmGCB product to hydrophobic charge induction chromatography (HCIC) and/or hydrophobic interaction chromatography (HIC); and subjecting the hmGCB product to one or more of anion

exchange chromatography, cation exchange chromatography, and size exclusion chromatography; to thereby obtain purified hmGCB.

In a preferred embodiment, chromatography material MEP HYPERCEL® is used for HCIC. In another preferred embodiment, chromatography material MACROPREP METHYL® is used for HIC.

In a preferred embodiment, the method includes using anion exchange chromatography. Preferably, anion exchange chromatography is performed using one or more of the following chromatography materials: Q SEPHAROSE FAST FLOW®, MACROPREP HIGH Q SUPPORT®, DEAE SEPHAROSE FAST FLOW®, AND MACRO-PREP DEAE®.

In a preferred embodiment, the method includes using cation exchange chromatography. Preferably, cation exchange chromatography is performed using one or more of the following chromatography materials: SP SEPHAROSE FAST FLOW®, SOURCE 30S®, CM SEPHAROSE FAST FLOW®, MACRO-PREP CM SUPPORT®, AND MACRO-PREP HIGH S SUPPORT®.

In a preferred embodiment, the method includes using size exclusion chromatography. Preferably, the size exclusion chromatography is performed using one or more of the following chromatography materials: SUPERDEX 200®, SEPHACRYL S-200 HR® AND BIO-GEL A 1.5M®.

In a preferred embodiment, the hmGCB is subjected to (in any order): anion exchange chromatography and cation exchange chromatography; anion exchange chromatography and size exclusion chromatography; cation exchange chromatography and size exclusion chromatography; anion exchange chromatography, cation exchange chromatography and size exclusion chromatography. Preferably, the hmGCB is subjected to all three of these chromatography steps in the following order: anion exchange chromatography, cation exchange chromatography and size exclusion chromatography.

In another aspect, the invention features a method of purifying hmGCB. The method includes: providing a harvested hmGCB product; subjecting the hmGCB product to hydrophobic charge induction chromatography (HCIC) and/or hydrophobic interaction chromatography (HIC); subjecting the HCIC and/or HIC purified hmGCB product to anion exchange chromatography; subjecting the anion exchange purified hmGCB to cation exchange chromatography; and, subjecting the cation exchange purified hmGCB to size exclusion chromatography, to thereby obtain purified hmGCB.

In a preferred embodiment, chromatography material MEP HYPERCEL® is used for HCIC. In another preferred embodiment, chromatography material MACROPREP METHYL® is used for HIC.

In a preferred embodiment, anion exchange chromatography is performed using one or more of the following chromatography materials: Q Sepharose Fast Flow®, MacroPrep High Q Support®, DEAE Sepharose Fast Flow®, and Macro-Prep DEAE®.

In a preferred embodiment, cation exchange chromatography is performed using one or more of the following chromatography materials: SP SEPHAROSE FAST FLOW®, SOURCE 30S®, CM SEPHAROSE FAST FLOW®, MACRO-PREP CM SUPPORT®, AND MACRO-PREP HIGH S SUPPORT®.

In a preferred embodiment, size exclusion chromatography is performed using one or more of the following chromatography materials: SUPERDEX 200®, SEPHACRYL S-200 HR® AND BIO-GEL A 1.5M®.

The term "high mannose glucocerebrosidase (hmGCB)" as used herein refers to glucocerebrosidase having at least one carbohydrate chain having four or more mannose residues from a precursor oligosaccharide. Preferably, the hmGCB has five, six, seven, eight or nine mannose residues from the precursor oligosaccharide chain. Most preferably, the hmGCB has five, eight or nine mannose residues from the precursor oligosaccharide chain.

The term "hmGCB preparation" refers to two or more hmGCB molecules.

The term "primary cell" includes cells present in a suspension of cells isolated from a vertebrate tissue source (prior to their being plated i.e., attached to a tissue culture substrate such as a dish or flask), cells present in an explant derived from tissue, both of the previous types of cells plated for the first time, and cell suspensions derived from these plated cells. The term secondary cell or cell strain refers to cells at all subsequent steps in culturing. That is, the first time a plated primary cell is removed from the culture substrate and replated (passaged), it is referred to herein as a secondary cell, as are all cells in subsequent passages. Secondary cells are cell strains which consist of secondary cells which have been passaged one or more times. A cell strain consists of secondary cells that: 1) have been passaged one or more times; 2) exhibit a finite number of mean population doublings in culture; 3) exhibit the properties of contact-inhibited, anchorage dependent growth (anchorage-dependence does not apply to cells that are propagated in suspension culture); and 4) are not immortalized. A "clonal cell strain" is defined as a cell strain that is derived from a single founder cell. A "heterogenous cell strain" is defined as a cell strain that is derived from two or more founder cells.

"Immortalized cells", as used herein, are cell lines (as opposed to cell strains with the designation "strain" reserved for primary and secondary cells), a critical feature of which is that they exhibit an apparently unlimited lifespan in culture.

The term "transfected cell" refers to a cell into which an exogenous synthetic nucleic acid sequence, e.g., a sequence which encodes a protein, is introduced. Once in the cell, the synthetic nucleic acid sequence can integrate into the recipient's cells chromosomal DNA or can exist episomally. Standard transfection methods can be used to introduce the synthetic nucleic acid sequence into a cell, e.g., transfection mediated by liposome, polybrene, DEAE dextran-mediated transfection, electroporation, calcium phosphate precipitation or microinjection. The term "transfection" does not include delivery of DNA or RNA into a cell by a virus.

The term "infected cell" or "transduced cell" refers to a cell into which an exogenous synthetic nucleic acid sequence, e.g., a sequence which encodes a protein, is introduced by a virus. Viruses known to be useful for gene transfer include an adenovirus, an adeno-associated virus, a herpes virus, a mumps virus, a poliovirus, a retrovirus, a Sindbis virus, a lentivirus and a vaccinia virus such as a canary pox virus.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

BRIEF DESCRIPTION OF THE DRAWING

FIG. 1 is a diagram showing the trimming of N-linked glycans as it occurs in the endoplasmic reticulum, the intermediate compartment and in the Golgi apparatus. The enzymes are numbered as follows: (1) α -glucosidase I; (2) α -glucosidase II; (3) ER mannosidase I; (4) ER mannosidase

II; (5) ER glucosyl transferase; (6) endomannosidase; (7) Golgi mannosidase IA, IB and IC; (8) GlcNAc transferase I; (9) Golgi mannosidase II. Δ : Glucose; \square : GlcNAc; \bullet : Mannose. Enzymes (3) and (7) are inhibited by kifunensine; enzyme (9) is inhibited by swainsonine.

DETAILED DESCRIPTION OF THE INVENTION

The invention is based, in part, on the discovery that inhibition of the removal of one or more mannose residues distal from the pentasaccharide core of a precursor oligosaccharide chain of glucocerebrosidase (GCB), results in high mannose glucocerebrosidase (hmGCB) that is efficiently targeted to mannose receptors. The removal of a mannose residue from the pentasaccharide core of a precursor oligosaccharide chain can be prevented by inhibiting or reducing the activity of one or more mannosidase enzymes, e.g., one or more class I processing mannosidase(s) and/or class II processing mannosidase(s). By preventing or inhibiting the removal of one or more mannose residues, hmGCB having at least one carbohydrate chain with four or more mannose residues from the precursor oligosaccharide chain can be obtained.

Gaucher disease is caused by a deficiency of GCB. GCB is required for degradation of glycosphingolipid glucocerebroside. In the absence of GCB, the glucocerebroside accumulates primarily in phagocytic cells, e.g., macrophages, and, ultimately, builds up in the liver, spleen and bone marrow.

Macrophages have mannose receptors. These receptors play a role in receptor-mediated endocytosis by these cells. hmGCB efficiently targets the mannose receptors on macrophages and improves the uptake of GCB (in the form of hmGCB) into these cells. By directing GCB (in the form of hmGCB) to the cells in which glucocerebroside accumulates, hmGCB can be used to hydrolyze glucocerebroside in the macrophages, thereby reducing the subsequent accumulation of this glycolipid in the liver, spleen and bone marrow of patients having Gaucher disease.

Glucocerebrosidase

Nucleotide sequence information is available for genes encoding glucocerebrosidase from various species. (See Horowitz et al. (1989) *Genomics* 4(1):87-96, disclosing the gene sequence (SEQ ID NO: 1) and amino acid sequence (SEQ ID NO: 2) of human glucocerebrosidase; Beutler et al. (1992) *Genomics* 12(4):795-800).

Mature human GCB has five potential N-linked glycosylation sites at Asn-19, Asn-59, Asn-146, Asn-270, and Asn-462. Glycosylation occurs at four of the five sites in human tissue derived GCB (Erickson et al. (1985) *J. Biol. Chem.* 260:14319-14324). Studies employing site-directed mutagenesis have demonstrated that the site at Asn-462 is never occupied (Berg-Fussman et al. (1993) *J. Biol. Chem.* 268:14861-14866). Approximately 20% of the released glycan chains from human placental GCB were shown to be of the high mannose type containing up to seven mannose-residues, whereas the majority of the glycan chains were of the complex type with sialylated biantennary and triantennary structures. (Takasaki et al. (1984) *J. Biol. Chem.* 259:10112-10117)

The first event in GCB N-glycosylation is the co-translational transfer in the lumen of the endoplasmic reticulum (ER) of Glc₃Man₂GlcNAc₂ from oligosaccharide-PP-dolichol to nascent peptide. The presence of the three glucose residues on the donor oligosaccharide allows for efficient

transfer to an acceptor asparagine by oligosaccharyl transferase. Following N-glycosylation, the glucose residues are rapidly removed from GCB during the folding process by ER glucosidases I and II. Two different ER mannosidases are each capable of hydrolyzing a single mannose residue from $\text{Man}_3\text{GlcNAc}_2$ to form two different isomers of $\text{Man}_2\text{GlcNAc}_2$ (see FIG. 1). Accessible glycans are then further processed in the Golgi to $\text{Man}_2\text{GlcNAc}_2$ by the removal of up to four $\alpha 1,2$ -linked mannose residues by Golgi mannosidase I. There are at least three different human genes encoding related Golgi mannosidase I isoforms (IA, IB, and IC) with slightly different substrate specificities and tissue expression but all are capable of trimming four mannose residues from $\text{Man}_3\text{GlcNAc}_2$ glycans to form $\text{Man}_2\text{GlcNAc}_2$ (Tremblay et al. (Jul. 27, 2000) *J. Biol. Chem.* [pub ahead of print]). They are located on chromosomes 6q22, 1p13, and 1p35-36 and their cDNA sequences are obtainable from GenBank as X74837, AF027156, and AF261655, respectively.

The final stage of processing that commits a glycan to the biosynthetic pathway for complex glycans requires the initial conversion of $\text{Man}_2\text{GlcNAc}_2$ to $\text{GlcNAcMan}_2\text{GlcNAc}_2$ by the action of GlcNAc transferase I, after which Golgi mannosidase II can catalyze the removal of two further mannose residues to yield $\text{GlcNAcMan}_2\text{GlcNAc}_2$. This is the substrate for glycan elongation by glycosyl transferases located in the trans Golgi and the trans Golgi network to form complex type chains.

If the high mannose chains transferred to GCB in the initial N-glycosylation step can be prevented from being processed to complex chains in the Golgi, then GCB with high mannose chains (hmgCB) will effectively target the mannose receptors on reticuloendothelial cells.

Cells

Primary and secondary cells to be transfected or infected can be obtained from a variety of tissues and include cell types which can be maintained and propagated in culture. For example, primary and secondary cells which can be transfected or infected include fibroblasts, keratinocytes, epithelial cells (e.g., mammary epithelial cells, intestinal epithelial cells), endothelial cells, glial cells, neural cells, formed elements of the blood (e.g., lymphocytes, bone marrow cells), muscle cells and precursors of these somatic cell types. Primary cells are preferably obtained from the individual to whom the transfected or infected primary or secondary cells are administered (i.e., an autologous cell). However, primary cells may be obtained from a donor (other than the recipient) of the same species (i.e., an allogeneic cell) or another species (i.e., a xenogeneic cell) (e.g., mouse, rat, rabbit, cat, dog, pig, cow, bird, sheep, goat, horse, monkey, baboon).

Primary or secondary cells of vertebrate, particularly mammalian, origin can be transfected or infected with an exogenous DNA sequence, e.g., an exogenous DNA sequence encoding a therapeutic protein, and produce an encoded therapeutic protein stably and reproducibly, both in vitro and in vivo, over extended periods of time. In addition, the transfected or infected primary and secondary cells can express the encoded product in vivo at physiologically relevant levels, cells can be recovered after implantation and, upon reculturing, to grow and display their preimplantation properties. Cells can be modified to reduce cell surface histo compatibility complex or foreign carbohydrate moieties to reduce immunogenicity, e.g., a universal donor cell.

Alternatively, primary or secondary cells of vertebrate, particularly mammalian, origin can be transfected or infected with an exogenous DNA sequence which includes a regulatory sequence. Examples of such regulatory sequences include one or more of: a promoter, an UAS, a scaffold attachment region or a transcription binding site. The targeting event can result in the insertion of the regulatory sequence of the DNA sequence, placing a targeted endogenous gene under their control (for example, by insertion of either a promoter or an enhancer, or both, upstream of the endogenous gene or regulatory region). Optionally, the targeting event can simultaneously result in the deletion of an endogenous regulatory sequence, such as the deletion of a tissue-specific negative regular sequence, of a gene. The targeting event can replace an existing regulatory sequence; for example, a tissue-specific enhancer can be replaced by an enhancer that has broader or different cell-type specificity than the endogenous elements, or displays a pattern of regulation or induction that is different from the corresponding nontransfected or noninfected cell. In this regard, the endogenous sequences are deleted and new sequences are added. Alternatively, the endogenous regulatory sequences are not removed or replaced but are disrupted or disabled by the targeting event, such as by targeting the exogenous sequences within the endogenous regulatory elements. Introduction of a regulatory sequence by homologous recombination can result in primary or secondary cells expressing a therapeutic protein which it does not normally express. In addition, targeted introduction of a regulatory sequence can be used for cells which make or contain the therapeutic protein but in lower quantities than normal (in quantities less than the physiologically normal lower level) or in defective form, and for cells which make the therapeutic protein at physiologically normal levels, but are to be augmented or enhanced in their content or production. Methods of activating an endogenous coding sequence are described in U.S. Pat. Nos. 5,641,670, 5,733,761 and 5,968,502, the contents of which are incorporated herein by reference.

The transfected or infected primary or secondary cells may also include a DNA sequence encoding a selectable marker which confers a selectable phenotype upon them, facilitating their identification and isolation. Methods for producing transfected primary or secondary cells which stably express the DNA sequence, clonal cell strains and heterogenous cell strains of such transfected cells, methods of producing the clonal and heterogenous cell strains, are known and described, for example, in U.S. Pat. Nos. 5,641,670, 5,733,761 and 5,968,502, the contents of which are incorporated herein by reference.

Transfected primary or secondary cells, can be made by electroporation. Electroporation is carried out at appropriate voltage and capacitance (and corresponding time constant) to result in entry of the DNA construct(s) into the primary or secondary cells. Electroporation can be carried out over a wide range of voltages (e.g., 50 to 2000 volts) and corresponding capacitance. Total DNA of approximately 0.1 to 500 μg is generally used.

Alternatively, known methods such as calcium phosphate precipitation, microinjection, modified calcium phosphate precipitation and polybrene precipitation, liposome fusion and receptor-mediated gene delivery can be used to transfect cells.

Processing of Glucocerebrosidase

Oligosaccharide assembly in cells which have not been treated to prevent removal of mannose residues usually proceeds as discussed below:

The oligosaccharide chains of GCB are attached to the polypeptide backbone by N-glycosidic linkages. N-linked glycans have an amide bond that connects the anomeric carbon (C-1) of a reducing-terminal N-acetylglucosamine (GlcNAc) residue of the oligosaccharide and a nitrogen of an asparagine (Asn) residue of the polypeptide.

Initiation of N-linked oligosaccharide assembly does not occur directly on the Asn residues of the GCB protein, but rather involves preassembly of a lipid-linked 14 sugar precursor oligosaccharide which is then transferred to the protein in the ER during or very soon after its translation from mRNA. A "precursor oligosaccharide" as used herein refers to the oligosaccharide chain involved in the initial steps in biosynthesis of carbohydrate chains. A "precursor oligosaccharide" can be an oligosaccharide structure which includes at least the following sugars: $\text{Man}_5\text{GlcNAc}_2$, for example, a precursor oligosaccharide can have the following structure: $\text{Glc}_3\text{Man}_5\text{GlcNAc}_2$, as shown in FIG. 1. The precursor oligosaccharide is synthesized while attached via a pyrophosphate bridge to a polyisoprenoid carrier lipid, a dolichol. This assembly involves at least six distinct membrane bound glycosyltransferases. Some of these enzymes transfer monosaccharides from nucleotide sugars, while others utilize dolichol-linked monosaccharides as sugar donors. After assembly of the lipid-linked precursor is complete, another membrane-bound enzyme transfers it to sterically accessible Asn residues which occur as part of the sequence -Asn-X-Ser/Thr.

Glycosylated Asn residues of newly-synthesized GCB transiently carry $\text{Glc}_3\text{Man}_5\text{GlcNAc}_2$, also referred to herein as an "unprocessed carbohydrate chain".

The processing of N-linked oligosaccharides is accomplished by the sequential action of a number of membrane-bound enzymes and begins immediately after transfer of the precursor oligosaccharide $\text{Glc}_3\text{Man}_5\text{GlcNAc}_2$ to the protein. The terms "processing", "trimming" and "modifying" are used interchangeably herein.

N-linked oligosaccharide processing can be divided into three stages: removal of the three glucose residues, removal of a variable number of mannose residues, and addition of various sugar residues to the resulting trimmed core.

The removal of the glucose residues in the first stage of processing involves removal of all three glucose residues to generate N-linked $\text{Man}_5\text{GlcNAc}_2$. This structure is also referred to herein as: $\text{Man}\alpha 1-2\text{Man}\alpha 1-2\text{Man}\alpha 1-3[\text{Man}\alpha 1-2\text{Man}\alpha 1-3(\text{Man}\alpha 1-2\text{Man}\alpha 1-6)\text{Man}\alpha 1-6]\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}$ (See FIG. 1, structure 9'). Processing normally continues to the second stage with removal of mannose residues.

Four of the mannose residues of the $\text{Man}_5\text{GlcNAc}_2$ moiety are bound by $\alpha 1,2$ linkages. Up to four of these $\alpha 1,2$ -linked mannose residues can be removed by mannosidase IA, IB and IC to generate N-linked $\text{Man}_{5-n}\text{GlcNAc}_2$.

Protein-linked $\text{Man}_5\text{GlcNAc}_2$ can then serve as a substrate for GlcNAc transferase I, which transfers a $\beta 1,2$ -linked GlcNAc residue from UDP-GlcNAc to the core $\alpha 1,3$ -linked mannose residue to form $\text{GlcNAcMan}_5\text{GlcNAc}_2$. Mannosidase II can then complete the trimming phase of the processing pathway by removing two mannose residues to generate a protein-linked oligosaccharide which contains within it a $\text{Man}_3\text{GlcNAc}_2$, the "pentasaccharide core". The structure $\text{GlcNAcMan}_3\text{GlcNAc}_2$ is then a substrate for GlcNAc transferase II, which can transfer a $\beta 1,2$ -linked GlcNAc residue to the $\alpha 1,6$ -linked mannose residue.

After the trimming phase, monosaccharides are sequentially added to the growing oligosaccharide chain by a series of membrane-bound Golgi glycosyltransferases, each of

which is highly specific with respect to the acceptor oligosaccharide, the donor sugar, and the type of linkage formed between the sugars. These can include distinct GlcNAc transferases (producing $\beta 1,2$; $\beta 1,4$; or $\beta 1,6$ linkages); galactosyltransferases (producing $\beta 1,4$; $\beta 1,3$; and $\alpha 1,3$ linkages); sialyltransferases (one producing $\alpha 2,3$ and another, $\alpha 2,6$ linkages); fucosyltransferases (producing $\alpha 1,2$; $\alpha 1,3$; $\alpha 1,4$ or $\alpha 1,6$ linkages); and a growing list of other enzymes responsible for a variety of unusual linkages. The cooperative action of these glycosyltransferases produces a diverse family of structures collectively referred to as "complex" oligosaccharides. These may contain two, three or four outer branches ("antennae") attached to the invariant core pentasaccharide, $\text{Man}_3\text{GlcNAc}_2$. These structures are referred to in terms of the number of their outer branches: biantennary (two branches), triantennary (three branches) or tetraantennary (four branches). The size of these complex glycans can vary.

Processing of High Mannose Glucocerebrosidase

hmGCB can be produced by reducing or preventing cellular carbohydrate modification (i.e., processing) of GCB. Carbohydrate modification can be prevented by allowing production of GCB under conditions which prevent the removal of at least one mannose residue distal to the pentasaccharide core of a precursor oligosaccharide chain of GCB. For example, one or more of the "trimming" stages during the removal of mannose residues from a precursor oligosaccharide can be prevented.

Cellular mannosidases fall into two broad classes: class 1 processing enzymes, which include ER mannosidase I, Golgi mannosidase IA, IB and IC and which hydrolyze $\alpha 1,2$ -linked mannose residues, and require Ca^{2+} for activity; and class 2 processing enzymes, which include ER mannosidase II, Golgi mannosidase II, cytosolic α -mannosidase, and lysosomal α -mannosidase and which have a broader substrate specificity and do not require Ca^{2+} for activity.

The trimming of mannose residues from the precursor oligosaccharide involves at least the following mannosidase enzymes: Golgi mannosidase IA, IB and IC, and Golgi mannosidase II. By inhibiting one or more of these mannosidases during N-linked oligosaccharide assembly in a cell, GCB can be produced which has at least one carbohydrate chain with one or more mannose residues in addition to the pentasaccharide core. For example, inhibition of both ER mannosidase I and Golgi mannosidase I can produce hmGCB with at least one carbohydrate chain (and preferably all chains) having at least eight mannose residues from the precursor oligosaccharide; inhibition of Golgi mannosidase II can produce hmGCB with at least one carbohydrate chain (and preferably all chains) having at least five mannose residues from the precursor oligosaccharide.

Trimming by a mannosidase can be inhibited, for example, by contacting the cell with a substance which prevents the removal of one or more mannose residues from a precursor oligosaccharide of GCB or by producing GCB in a cell which does not produce or produces at deficient levels at least one mannosidase, or in a cell which produces a mutated and/or inactive mannosidase. For example, the cell can be a knockout for at least one mannosidase, can express at least one antisense mannosidase molecule or can be dominant negative for at least one mannosidase.

Substances which Prevent Removal of Mannose Residues

A substance which prevents the removal of one or more mannose residues from a precursor oligosaccharide of GCB can be used to produce an hmGCB preparation. For example, a cell which expresses GCB can be contacted with

a substance which prevents the removal of one or more α 1,2 mannose residues of a precursor oligosaccharide of GCB, and/or removal of an α 1,3 mannose residue of a precursor oligosaccharide of GCB, and/or removal of an α 1,6 mannose residue of a precursor oligosaccharide of GCB. Preferably, the substance is a mannosidase inhibitor, e.g., a class 1 processing mannosidase inhibitor or a class 2 processing mannosidase inhibitor.

Cellular mannosidases fall into two broad classes on the basis of protein sequence homologies (Moremen et al. (1994) *Glycobiology* 4:113-125). These two classes are mechanistically different. Class 1 enzymes, which include ER mannosidase I and Golgi mannosidase I isoforms, have a mass of about 63-73 kDa, hydrolyze α 1,2-linked mannose residues and require Ca^{2+} for activity. Class 1 processing mannosidases can be blocked, for example, by treatment with a substrate mimic, e.g., a pyranose analog of mannose. For example, class 1 processing mannosidases can be blocked by treatment with one or more of the following enzymatic inhibitors: kifunensine, deoxymannojirimycin, or a combination thereof. Class 2 enzymes, which include ER mannosidase I, Golgi mannosidase II, cytosolic α -mannosidase, and lysosomal α -mannosidase, have a greater mass of about 107-136 kDa, do not require Ca^{2+} for activity and have a broader substrate specificity. Class 2 processing mannosidases can be blocked, for example, by treatment with furanose transition state analogues of the mannosylation (Daniels et al. (1994) *GlycoBiol.* 4:551-566). For example, class 2 processing mannosidases can be blocked by treatment with one or more of the following inhibitors: swainsonine, 6-deoxy-DIM, 6-deoxy-6-fluoro-DIM, mannostatin A, or combinations thereof.

Kifunensine can be used as an inhibitor of the endoplasmic reticulum mannosidase I and/or Golgi mannosidase IA and/or IB and/or IC; deoxymannojirimycin can be used as an inhibitor of ER mannosidase I, ER mannosidase II and/or of Golgi mannosidase IA and/or IB and/or IC; swainsonine can be used as an inhibitor of Golgi mannosidase II; and mannostatin A can be used as an inhibitor of Golgi mannosidase II.

Use of a mannosidase inhibitor can inhibit the processing of a carbohydrate chain of GCB past a certain stage of mannose residue trimming during oligosaccharide assembly. For example, contacting a cell with kifunensine can inhibit trimming of any, or one, two, three, or four of the mannose residues of a precursor oligosaccharide.

Processing α -mannosidases can be blocked by treatment of cells with one or more of the following enzyme inhibitors:

Kifunensine, an inhibitor of the endoplasmic reticulum I and Golgi mannosidase I enzymes (Weng and Spiro (1993) *J. Biol. Chem.* 268:25656-25663; Elbein et al. (1990) *J. Biol. Chem.* 265:15599-15605).

Swainsonine, an inhibitor of the Golgi mannosidase II enzyme (Tulsiani et al. (1982) *J. Biol. Chem.* 257:7936-7939).

Deoxymannojirimycin, an inhibitor of both endoplasmic reticulum mannosidases I and II and of Golgi mannosidase I (Weng and Spiro (1993) *J. Biol. Chem.* 268:25656-25663; Tremblay and Herscovics (2000) *J. Biol. Chem.* July 27; [epub ahead of print]).

DIM (1,4-di-deoxy-1,4-imino-D-mannitol), an inhibitor of Golgi mannosidase II (Palamarzyk et al. (1985) *Arch. Biochem. Biophys.* 243:35-45).

6-Deoxy-DIM and 6-deoxy-6-fluoro-DIM, inhibitors of Golgi mannosidase II (Winchester et al. (1993) *Biochem J.* 290:743-749).

Mannostatin A, an inhibitor of Golgi mannosidase II (Tropea et al. (1990) *Biochemistry* 29:10062-10069).

Various mannosidase inhibitors can be selected by their ability to penetrate particular cell types as well as by the inhibitory potency of the mannosidase inhibitor. For example, swainsonine is rapidly internalized by cultured fibroblasts in a time- and concentration-dependent manner. Swainsonine is also a potent inhibitor of a class 2 mannosidase, e.g., Golgi mannosidase II. Thus, swainsonine can be used to produce hmGCB in cultured fibroblasts, e.g., hmGCB having at least one carbohydrate chain which has at least four or five mannose residues of the precursor oligosaccharide. In addition, kifunensine is readily taken up by cultured fibroblasts and is a potent inhibitor of class 1 mannosidases, e.g., ER mannosidase I and Golgi mannosidase I. Thus, kifunensine can be used to produce hmGCB in cultured fibroblasts, e.g., hmGCB having at least one carbohydrate chain which has at least four, five, six, seven, eight or nine mannose residues of the precursor oligosaccharide.

Preferably, the mannosidase inhibitor is present at a concentration of 0.025 to 20.0 $\mu\text{g/ml}$, 0.05 to 10 $\mu\text{g/ml}$, 0.05 to 5 $\mu\text{g/ml}$, preferably between about 0.1 to 2.0 $\mu\text{g/ml}$. For example, a class 1 processing mannosidase inhibitor can be present at a concentration between about 0.025 to 20.0 $\mu\text{g/ml}$, 0.05 to 10 $\mu\text{g/ml}$, 0.05 to 5 $\mu\text{g/ml}$, preferably between about 0.1 to 2.0 $\mu\text{g/ml}$; a class 2 processing mannosidase inhibitor can be present at a concentration between about 0.025 to 20.0 $\mu\text{g/ml}$, 0.05 to 10 $\mu\text{g/ml}$, 0.05 to 5 $\mu\text{g/ml}$, preferably between about 0.1 to 2.0 $\mu\text{g/ml}$; each of the class 1 processing and class 2 processing mannosidase inhibitors can be present at a concentration between about 0.025 to 20.0 $\mu\text{g/ml}$, 0.05 to 10 $\mu\text{g/ml}$, 0.05 to 5 $\mu\text{g/ml}$, preferably between about 0.1 to 2.0 $\mu\text{g/ml}$; or the total concentration of the class 1 processing and class 2 processing mannosidase inhibitors present can be between about 0.025 to 40.0 $\mu\text{g/ml}$, 0.05 to 20 $\mu\text{g/ml}$, 0.05 to 10 $\mu\text{g/ml}$, preferably between about 0.1 to 5.0 $\mu\text{g/ml}$.

The cell can be contacted with a mannosidase inhibitor by, for example, culturing the cell on medium which includes at least one mannosidase inhibitor.

Mannosidase Mutant Cell

Mannosidase Knockout Cell

Permanent or regulated inactivation of mannosidase gene expression can be achieved by targeting to a mannosidase locus with a transfected plasmid DNA construct or a synthetic oligonucleotide. The plasmid construct or oligonucleotide can be designed to several forms. These include the following: 1) insertion of selectable marker genes or other sequences within an exon of a mannosidase gene; 2) insertion of exogenous sequences in regulatory regions of non-coding sequence; 3) deletion or replacement of regulatory and/or coding sequences; and, 4) alteration of a protein coding sequence by site specific mutagenesis.

In the case of insertion of a selectable marker gene into coding sequence, it is possible to create an in-frame fusion of an endogenous mannosidase exon with the mannosidase exon engineered to contain, for example, a selectable marker gene. In this way following successful targeting, the endogenous mannosidase gene expresses a fusion mRNA (mannosidase sequence plus selectable marker sequence). Moreover, the fusion mRNA would be unable to produce a functional mannosidase translation product.

In the case of insertion of DNA sequences into regulatory regions, the transcription of a mannosidase gene can be silenced by disrupting the endogenous promoter region or any other regions in the 5' untranslated region (5' UTR) that is needed for transcription. Such regions include, for

example, translational control regions and splice donors of introns. Secondly, a new regulatory sequence can be inserted upstream of the mannosidase gene that would render the mannosidase gene subject to the control of extracellular factors. It would thus be possible to down-regulate or extinguish mannosidase gene expression as desired for optimal hmGCB production. Moreover, a sequence which includes a selectable marker and a promoter can be used to disrupt expression of the endogenous sequence. Finally, all or part of the endogenous mannosidase gene could be deleted by appropriate design of targeting substrates.

In order to create a cell which includes a knockout of at least one chromosomal copy of the human Golgi mannosidase IA, IB or IC gene, the genomic DNA comprising at least the 5' portion of the gene (including regulatory sequences, 5' UTR, coding sequence) is isolated. For example, the GenBank sequence, Accession No.: NM005907 (human), can be used to generate a probe for Golgi mannosidase IA or Accession Nos.: AAF97058 can be used to generate a probe for Golgi mannosidase IB or IC using polymerase chain reaction (PCR). Oligonucleotides for PCR can be designated based upon the GenBank sequence. The resulting probe can hybridize to the single copy Golgi mannosidase IA, IB or IC gene. This probe can then be used to screen a commercially available recombinant phage library (e.g., a library made from human genomic DNA) to isolate a clone comprising all or part of the mannosidase I structural genes. Once a recombinant clone comprising a mannosidase regulatory and/or coding sequence is isolated, specific targeting plasmids designed to achieve the inactivation of mannosidase gene expression can then be constructed. Inactivation of mannosidase activity results from the insertion of exogenous DNA into regulatory or coding sequences to disrupt the translational reading frame. Inactivation of the enzyme can also be the result of disruption of mRNA transcription or mRNA processing, or by deletion of endogenous mannosidase regulatory or coding sequences.

The nucleic acid sequence of other class 1 and class 2 processing mannosidase are also available, for example, in GenBank. Using the methods described above for Golgi mannosidase IA, IB or IC, a knockout cell for other class 1 and/or class 2 processing mannosidases can be produced.

A mannosidase knockout cell can be used, for example, in gene therapy. A knockout cell can be administered to a subject, e.g., a subject having Gaucher disease, such that the cell produces hmGCB in vivo.

Antisense Mannosidase Nucleic Acid Sequences

Nucleic acid molecules which are antisense to a nucleotide encoding a mannosidase, e.g., a class 1 processing or class 2 processing mannosidase, can be used as an inactivating agent which inhibits expression of a mannosidase. For example, Golgi mannosidase IA, Golgi mannosidase IB, Golgi mannosidase IC, and/or Golgi mannosidase II expression can be inhibited by an antisense nucleic acid molecule. An "antisense" nucleic acid includes a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a mannosidase, e.g., complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. Accordingly, an antisense nucleic acid can form hydrogen bonds with a sense nucleic acid. The antisense nucleic acid can be complementary to an entire mannosidase coding strand, or to only a portion thereof. For example, an antisense nucleic acid molecule which antisense to the "coding region" of the coding strand of a nucleotide sequence encoding a mannosidase can be used.

As the coding strand sequences encoding various mannosidases are disclosed in, for example, Bause (1993) *Eur. J. Biochem.* 217(2):535-540; Gonzalez et al. (1999) *J. Biol. Chem.* 274(30):21375-21386; Misago et al. (1995) *Proc. Natl. Acad. Sci. USA* 92(25): 11766-11770; Tremblay et al. (1998) *Glycobiology* 8(6):585-595; Tremblay et al. (2000) *J. Biol. Chem.* July 27:[epub ahead of print], antisense nucleic acids can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule can comprise sequence complementary to the entire coding region of a mannosidase mRNA, but more preferably is an oligonucleotide which is complementary to only a portion of the coding or noncoding region of a mannosidase mRNA. For example, the antisense oligonucleotide can comprise sequence complementary to the region surrounding the translation start site of a mannosidase mRNA. An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, or more nucleotides in length. An antisense nucleic acid can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (e.g., an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, e.g., phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl)uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylquenosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxymethyl-2-thiouracil, beta-D-mannosylquenosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N-6-isopentenyladenine, uracil-5-oxyacetic acid (v), ybutoxosine, pseudouracil, quenosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methyl-ester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (i.e., RNA transcribed from the inserted nucleic acid will be of an antisense orientation relative to a target nucleic acid of interest).

Purification of hmGCB

The term "purified" hmGCB, as used herein, refers to hmGCB that is substantially free of cellular material when produced by a cell which expresses GCB. The language "substantially free of cellular material" includes preparations of hmGCB in which the protein is separated from cellular components of the cells in which it is produced. In one embodiment, the language "substantially free of cellular material" includes preparations of hmGCB having less than about 30% (by dry weight) of non-GCB protein (also referred to herein as a "protein impurity" or "contaminating protein"), more preferably less than about 20% of non-GCB protein, still more preferably less than about 10% of non-GCB protein, and most preferably less than about 5%

non-GCB protein. When the hmGCB is obtained (i.e., harvested) from culture media, it is also preferably substantially free of a component of the culture medium, i.e., components of the culture medium represent less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the dry weight of the protein preparation.

Various methods can be used to harvest hmGCB from culture media. The term "harvested hmGCB" as used herein refers to hmGCB obtained from culture media or from a cell. For example, one of the following alternatives can be used to prepare the harvested hmGCB prior to a purification procedure. These can include: 1) filtering the fresh harvest; 2) filtering the fresh harvest and freezing, e.g., at about -20° C. to -80° C., the filtered product until ready for processing (at which time it can be thawed and, optionally, filtered); 3) filtering the fresh harvest, concentrating filtered product (e.g., by about 8 to 10 fold), and then, optionally, filtering again; 4) filtering the fresh harvest, concentrating filtered product (e.g., by about 8 to 10 fold), optionally, filtering again, and then freezing, e.g., at about -20° C. to -80° C., until ready for processing (at which time it can be thawed and, optionally, filtered). Variations of these alternatives can also be performed. For example, when the harvested product or concentrated harvested product is frozen, different harvests can be pooled after thawing and filtered. In addition, for harvested or concentrated harvested product, the product can be held at a cooling temperature, e.g., about 2° C. to 8° C., for short periods of time, e.g., about 1 to 3 days, preferably 1 day, prior to purification. The harvested product held at the cooling temperature can be pooled prior to purification.

When a concentration of harvest is performed, an ultra-filtration membrane with a 5,000 to 50,000 mw cutoff, preferably a 10,000 to 30,000 mw cutoff, can be employed. Filter clarification will typically employ a 1.2 µm/0.5 µm prefilter, followed by a 0.2 µm final filter.

HmGCB can be purified by the following purification techniques. For example, hydrophobic charge induction chromatography (HCIC) can be used to purify the hmGCB preparation. Alternatively, hydrophobic interaction chromatography (HIC) can be used to purify the hmGCB preparation. Both HCIC and HIC are described below.

HCIC or HIC can be used alone or in combination with one or more ion exchange steps. Ion exchange steps that can be used in combination with an HCIC or HIC step (either before or after HCIC or HIC) include the use of anion exchange and/or cation exchange chromatography. Generally known commercially available anion exchange supports used in the purification of proteins bear quaternary ammonium functional groups. Preferred matrices for use in the present process are agarose or cellulose based matrices such as microcrystalline cellulose or cross-linked agaroses. Also particularly preferred are those matrices bearing diethyl aminoethyl, triethyl aminomethyl, or trimethyl aminomethyl functional groups. A particularly preferred anion exchange matrix is trimethyl aminomethyl crosslinked agarose, which is commercially available, e.g., Q-Sepharose Fast Flow® (Pharmacia). Generally known commercially available cation exchange supports that may be used in the purification of proteins bear acidic functionalities, including carboxy and sulfonic acids. Matrices containing the cation functionalities include various forms of celluloses and polystyrene based matrices. For example, weak cation exchangers known in the art include, but are not limited to, Carboxymethyl-Sepharose® and Carboxymethyl-Cellulose®. Strong cation exchangers known in the art include, but are not limited to,

sulfonated polystyrenes (AG 50W®, Bio-Rex 70®), sulfonated celluloses (SP-Sephadex®), and sulfonated Sepharoses (S-Sepharose®). A particularly preferred cation exchange matrix is S-Sepharose Fast Flow® (Pharmacia).

The chromatographic step involving these matrices is most preferably conducted as a column chromatography step or in alternative a batch absorptive technique, which optionally can be performed at a temperature between 25° C. to 40° C. Preferably, a salt is added to a washing or eluting buffer to increase the ionic strength of the buffer. Any of the salts conventionally used may be employed for this purpose as can be readily determined by one skilled in the art, with NaCl being one of the most frequently and conveniently used salts.

A conventional gel filtration step can also be used in combination with the HCIC or HIC chromatography process step. Representative examples of these matrices are polydextrans cross linked with acrylamides, such as composite hydrophilic gels prepared by covalently cross linking allyl dextran with N,N'-methylene bisacrylamide and crosslinked cellulose or agarose gels. Commercially available crosslinked dextran-acrylamides are known under the trade name Sephacryl® and are available from Pharmacia. Commercially available crosslinked dextran-agarose resins are known under the trade name Superdex®, available from Pharmacia. A preferred Superdex® gel is Superdex 200®. Examples of crosslinked cellulose gels are those commercially available cross linking porous cellulose gels, e.g., GLC 300® or GLC 1,000®, that are available from Amicon Inc. Silica based resins such as TSK-Gel SW®, available from TosohHaas can be utilized. Polymer based resins such as TSK-Gel PW®, TSK Alpha Series®, Toyopearl HW packings® (copolymerization of ethylene glycol and methyl acrylate polymers) are also available from TosohHaas.

Preferably, HCIC or HIC can be combined with one or more of these ion exchange steps. When a combination of HCIC or HIC and various ion exchange or gel filtration steps are used, they can be performed in any order. For example, as described below a four step procedure can be followed which includes HCIC using hydrophobic charge induction chromatography material MEP HYPERCEL® or HIC using hydrophobic interaction chromatography material MacroPrep Methyl®, then ion-exchange chromatography resins Q SEPHAROSE FAST FLOW®, SP SEPHAROSE FAST FLOW®, and lastly size-exclusion chromatography resin SUPERDEX 200®. Several of these procedures are set forth in more detail below.

MEP Hypercel Chromatography

MEP (mercaptoethylpyridine) Hypercel® (BioSeptra. Life Technologies) can be used for HCIC. It is a resin consisting of NEP linked to a regenerated cellulose bead of high porosity (80-100 microns). The functional group (MEP), consisting of a hydrophobic tail and an ionizable head group, is uncharged at neutral pH and can bind certain protein ligands based on hydrophobic interaction at a physiological ionic strength. Elution is accomplished by decreasing pH to 4 to 5, at which MEP is positively charged, and the protein elutes from the column due to electrostatic repulsion. For example, prepared harvest or harvest concentrate can be applied directly to the MEP column equilibrated with 25 mM sodium phosphate, pH 6.8, containing 180 mM sodium chloride and 2 mM DTT. Optionally, the column can then be washed with equilibration buffer containing 25 mM sodium caprylate until the absorbance at 280 nm (A280) stabilizes. The hmGCB can be eluted from the column with 50 mM sodium acetate, 2 mM DTT, pH 4.7, and the peak as monitored at 280 nm can be collected.

MacroPrep Methyl Chromatography

An alternative to MEP Hypercel® is MacroPrep Methyl®, which is a hydrophobic interaction chromatography (HIC) resin. This resin consists of a methyl functional group attached to a bead composition of macroporous copolymerized glycol methacrylate and diethylene glycol dimethacrylate. For example, MacroPrep Methyl® (BioRad) chromatography can be performed as follows. The pH of the harvest or harvest concentrate is adjusted to 5.6, and ammonium sulfate is added to 0.70 M final concentration. The prepared harvest can be applied to the MacroPrep Methyl® column, which has been equilibrated in 0.70 M ammonium sulfate, 10 mM MES, pH 5.6. After application of the load, the column is washed with equilibrated buffer until the A280 returns to baseline. The hmGCB can be eluted with 10 mM MES, pH 5.6. The eluted hmGCB can be ultrafiltered and/or diafiltered in preparation for steps such as an ion exchange step such as Q Sepharose chromatography, SP Sepharose chromatography and/or Superdex 200 Chromatography.

Q Sepharose Chromatography

Q Sepharose Fast Flow® (Amersham Pharmacia) is a relatively strong anion exchange chromatography resin. The functional substituent is a quaternary amine group, which is positively charged over the working pH range of 2 to 12. Proteins with a net negative charge at the working pH will tend to bind to the resin at a relatively low ionic strength and can be eluted at higher ionic strength or lower pH. HmGCB does not bind to Q Sepharose at approximately pH 6 and low ionic strength, but impurities do bind, thereby purifying the sample. For example, the following protocol can be used to purify hmGCB in the sample by Q Sepharose Fast Flow® chromatography. Under appropriate conditions, hmGCB flows through this column, so the product is found in the flowthrough/wash fraction. Sodium phosphate (250 mM, pH 6) is added to the MEP elution pool prepared as described above to a final concentration of 25 mM, and the pH of the pool is adjusted to pH 6 with NaOH (and HCl if necessary). The conductivity is adjusted to 2.5±0.1 mS/cm by dilution with water or by ultrafiltration/diafiltration using 25 mM sodium phosphate, 2 mM DTT, at approximately pH 6. The material is then filtered and applied to a column of Q Sepharose Fast Flow® which has been equilibrated in 25 mM sodium phosphate, 2 mM DTT, pH 6.0. After application of the load, the column is washed with equilibration buffer until the A280 reaches baseline. The flowthrough/wash fraction can then be processed through another column, e.g., SP Sepharose Fast Flow® column, shortly thereafter, e.g., within 24 hours, or frozen and stored at about -20° C. to -80° C. prior to further processing.

Other strong anion exchange resins, such as Macro-Prep High Q Support® (BioRad) can be used in place of Q Sepharose. A weaker anion exchange resin such as DEAE Sepharose Fast Flow® (Pharmacia) or Macro-Prep DEAE® (BioRad) can also be used. The column is equilibrated in buffer, e.g., 25 mM sodium phosphate, pH 6. The pH of the sample is adjusted to pH 6 and the conductivity is adjusted by dilution or diafiltration to a relatively low ionic strength, which allows impurities to bind to the column and hmGCB to flow through. The sample is applied and the column is washed with equilibration buffer. Impurities are still bound to the column, and can be eluted with application of salt, e.g., sodium chloride or potassium chloride, or application of a lower pH buffer, or a combination of increased salt and lower pH.

The hmGCB can also be allowed to bind the anion exchange column during loading by decreasing the salt

concentration in the load or by running the column at a higher pH, or by a combination of both decreased salt and higher pH.

SP Sepharose Chromatography

SP Sepharose Fast Flow® (Amersham Pharmacia) is a relatively strong cation exchange chromatography resin. The functional substituent is a charged sulfonic acid group, which is negatively charged over a working pH range of 2 to 12. Proteins with a net positive charge at the working pH will tend to bind to the resin at a relatively low ionic strength and can be eluted at higher ionic strength or higher pH. HmGCB binds to SP Sepharose at approximately pH 6 and intermediate ionic strength (e.g., 6.5 mS/cm) and can be eluted at higher ionic strength (e.g., 10.7 mS/cm). Impurity proteins remain bound to SP Sepharose under conditions of hmGCB elution, thereby purifying the hmGCB in the sample. For example, the following protocol can be used to purify hmGCB by SP Sepharose Fast Flow® chromatography. Sodium chloride (2.0 M stock) is added to the Q Sepharose® flowthrough/wash until the conductivity is 6.3 mS/cm. The pH is checked and readjusted to pH 6.0 if necessary. Then, addition of sodium chloride stock is continued until the conductivity is 6.5 mS/cm. The material is filtered and applied to a column of SP Sepharose Fast Flow®, which has been equilibrated with 25 mM sodium phosphate, 44 mM sodium chloride, pH 6.0. After application of the load, the column is washed with equilibration buffer until the baseline is reached and eluted with 25 mM sodium phosphate, 84 mM sodium chloride, pH 6.0. HmGCB is found in the elution fraction.

Another cation exchange resin, e.g., Source 30S® (Pharmacia), CM Sepharose Fast Flow® (Pharmacia), Macro-Prep CM Support® (BioRad) or Macro-Prep High S Support® (BioRad), can be used as an alternative to SP Sepharose. The hmGCB can bind to the column at approximately pH 6 and low to intermediate ionic strength, such as 4 to 7 mS/cm. A buffer, e.g., 10 mM sodium citrate, pH 6.0, 10 mM MES, pH 6.0, 25 mM sodium phosphate, pH 6.0, or other buffer with adequate buffering capacity at pH 6.0 can be used to equilibrate the column. The ionic strength of the sample is adjusted by dilution or diafiltration to a level which will accommodate binding to the column. The sample is applied to the column and the column is washed after the load to remove unbound material. A salt, e.g., sodium chloride or potassium chloride, can be used to elute the hmGCB from the column. Alternatively, the hmGCB can be eluted from the column with a buffer of higher pH or a combination of higher salt concentration and higher pH.

The hmGCB can also be made to flow through the cation exchange column during loading by increasing the salt concentration in the equilibration buffer and in the sample load, by running the column at a higher pH or by a combination of both increased salt and higher pH.

Superdex 200 Chromatography

Superdex 200 prep grade® (Amersham Pharmacia) is used for size exclusion chromatography of hmGCB, whereby molecules are separated by size, molecular mass, Stokes radius or hydrodynamic volume. Superdex 200 is composed of dextran covalently cross linked to agarose and has a fractionation range of 10,000 to 60,000 molecular weight for globular proteins. For example, the following protocol can be used to purify hmGCB by Superdex 200® chromatography. The SP elution pool is concentrated by ultrafiltration using a 10,000 mw cutoff membrane. The concentrated pool is filtered, then applied to a Superdex 200 prep grade® column which has been equilibrated in 50 mM sodium citrate, pH 6.0. The A280 of the column effluent in

the initial fractions is collected and, for example, an 8 to 16% SDS polyacrylamide gel is run to determine pooling of fractions. Pooling may be decided based on visual inspection of the silver-stained gel.

Other size exclusion chromatography resins such as Sephacryl S-200 HR®, Bio-Gel A 1.5 m®, or TosohHaas TSK Gel resins can also be used to purify hmGCB. The buffer used for size exclusion chromatography of hmGCB is 50 mM sodium citrate, pH 6.0. Other buffers can also be used such as 25 mM sodium phosphate, pH 6.0 containing 0.15 M sodium chloride. The pH of the buffer can be between pH 5 and pH 7 and should have sufficient ionic strength to minimize ionic interactions with the column.

Variations of pH, buffer and/or salt concentration in any of the purification protocols described above can be performed by routine methods to achieve the desired purified product.

Assays for Determining Macrophage Uptake and Cellular Targeting of hmGCB

The uptake efficiency of hmGCB by macrophages can be determined by assaying, e.g., protein levels and/or enzyme activity in macrophages. For example, as described in the Examples below and in Dimant et al. (1987) *J. Leukocyte Biol.* 42:485-490, an *in vitro* assay using a macrophage cell line can be used to determine absolute and mannose receptor specific uptake of hmGCB.

In addition, *in vivo* comparison of uptake of hmGCB and GCB by liver cells can be determined as described, for example, in Friedman et al. (1999) *Blood* 93:2807-2816. Briefly a mouse model can be injected with hmGCB or GCB, and then sacrificed shortly thereafter. The liver of the animal can then be used to prepare a suspension of liver cells, e.g., parenchymal cells, Kupffer cells, endothelial cells and hepatocytes. The cells can then be separated, identified by morphology and the protein levels and/or enzymatic activity of hmGCB and GCB in the various liver cell types can be determined. Alternatively, immunohistochemical detection may be used to localize hmGCB to a specific cell or cell type in tissue of treated animals.

Pharmaceutical Compositions

High mannose glucocerebrosidase (hmGCB) can be incorporated into pharmaceutical compositions suitable for administration to a subject, e.g., a human. The composition can include a sufficient dosage of hmGCB to treat a subject having Gaucher disease. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, excipients, dispersion media, coatings, antibacterial and antifungal agents, isotonic and adsorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, such media can be used in the compositions of the invention. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, and subcutaneous administration. Preferably, the route of administration is intravenous. Solutions or suspensions used for parenteral application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or

sodium bisulfite; chelating agents such as ethylenediamine-tetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders, e.g., lyophilized preparations, for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL® (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged stability of the injectable compositions can be brought about by including in the composition an agent which delays adsorption, for example, aluminum monostearate, human serum albumin and gelatin.

Sterile injectable solutions can be prepared by incorporating the hmGCB in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying, e.g., lyophilization, which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811.

Treatment of Gaucher Disease

HmGCB, e.g., any hmGCB molecule or preparation described herein, can be used to treat a subject having Gaucher disease. Alternatively, any mannosidase knockout cell described herein, can be introduced into a subject having Gaucher disease to deliver hmGCB to the subject. Various routes of administration and various sites can be used. Once implanted in individual, the knockout cell can produce hmGCB.

Preferably, the knockout cells used will generally be patient-specific genetically engineered cells. It is possible, however, to obtain cells from another individual of the same species or from a different species. Use of such cells might require administration of an immunosuppressant, alteration of histocompatibility antigens, or use of a barrier device to prevent rejection of the implanted cells.

Gaucher disease is an autosomal recessive lysosomal storage disorder characterized by a deficiency in the lysosomal enzyme, glucocerebrosidase (GCB). GCB hydrolyzes the glycolipid glucocerebroside that is formed after degradation of glycosphingolipids in the membranes of white blood cells and red blood cells. If GCB hydrolysis is insufficient then glucocerebroside can accumulate in macrophages (Gaucher cells), causing anemia, thrombocytopenia, organomegaly and major bone problems.

There are several types of Gaucher disease including Gaucher type 1, type 2 and type 3, which can arise due to various mutations in the GCB gene. A "therapeutically effective amount" of hmGCB, i.e., a dosage of hmGCB sufficient to treat Gaucher disease, can be given to a subject having this disorder. The term "treat" as used herein refers to reducing or inhibiting one or more symptoms of Gaucher disease. Symptoms of Gaucher disease type 1 include: skeletal complications such as bone pain, bone lesions, osteopenia, osteonecrosis, avascular necrosis and pathological fractures; anemia; hepatosplenomegaly; splenic nodules and liver dysfunction; thrombocytopenia; and/or delayed growth and pubertal development. Symptoms of Gaucher disease type II include the symptoms of Gaucher type I as well as neck rigidity, apathy, catatonia, strabismus, increased deep reflex and laryngeal spasm. Symptoms of Gaucher disease type III are similar to Gaucher type II except milder and later in onset.

A therapeutically effective amount of hmGCB can be determined on an individual basis and will be based, at least in part, on consideration of the size of the patient, the agent used, the type of delivery system used, the time of administration relative to the severity of the disease, and whether a single, multiple, or a controlled release dose regimen is employed. Preferably, the dosage of hmGCB sufficient to treat Gaucher disease is less than the dosage of human tissue derived or human placenta derived GCB, or GCB produced by cells in vitro and then trimmed to expose core mannose residues.

Treatment of Other Lysosomal Storage Diseases

Generally, the invention described herein can be used to produce proteins for targeting any cells that express mannose receptors on their surface. Thus, the invention described herein can be used to treat any disorder in which it is desirable to target a protein for treatment to a mannose receptor-expressing cell. For example, the invention described herein can also be applied to other lysosomal storage enzymes and other lysosomal storage diseases in which cells, e.g., the cells of reticuloendothelial origin, accumulate undigested substrate. Reticuloendothelial cells include macrophages, Kupffer cells in the liver and histio-

cytes in the spleen. Such lysosomal storage diseases include, but are not limited to, Farber disease and Neimann-Pick disease.

Farber disease is an autosomal recessive lysosomal storage disorder characterized by a deficiency in acid ceramidase. Ceramidases are enzymes responsible for degradation of ceramide. If ceramide degradation is insufficient then ceramide accumulates leading to granuloma formation and histiocytic response. (Moser, H. W. Ceramidase deficiency: Farber lipogranulomatosis; In: *The Metabolic and Molecular Basis of Inherited Disease* (C. R. Scriver, A. L. Beaudet, W. S. Sly and D. Valle, Eds.), Seventh edition, pp. 2589-2599, McGraw-Hill Inc., New York (1995))

There are several types of Farber disease including Farber type 1, type 2, type 3, type 4, and type 5 which differ in severity and sites of major tissue involvement. There is also type 6 and type 7 Farber disease. High mannose acid ceramidase can be given to a subject having Farber disease to treat, i.e., alleviate or reduce at least one symptom, of the disease. Symptoms of Farber disease type 1 include: swelling of the joints (particularly the interphalangeal, metacarpal, ankle, wrist, knee and elbow); palpable nodules in relation to the affected joints and over pressure points, a hoarse cry that may progress to aphonia, feeding and respiratory difficulty, poor weight gain and intermittent fever. The symptoms usually occur between ages two weeks and four months. Symptoms of Farber type 2 and type 3 include: subcutaneous nodules, joint deformities, and laryngeal involvement. These subjects survive longer than subjects having Farber type 1. Farber disease type 5 symptoms include psychomotor deterioration beginning at one to two and half years of age.

Neimann-Pick disease type A and type B are an autosomal recessive lysosomal storage disorder characterized by a deficiency acid sphingomyelinase. Acid sphingomyelinase is an enzyme responsible for degradation of sphingomyelin. If sphingomyelinase is deficient, sphingomyelin and other lipids can accumulate in the monocyte-macrophage system. (Schuman, E. H. and Desnick, R. J. Neimann-Pick Disease types A and B: acid sphingomyelinase deficiencies; In: *The Metabolic and Molecular Basis of Inherited Disease* (C. R. Scriver, A. L. Beaudet, W. S. Sly and D. Valle, Eds.), Seventh edition, pp. 2589-2599, McGraw-Hill Inc., New York (1995))

There are several types of Neimann-Pick disease including type A and type B. High mannose acid sphingomyelinase can be given to a subject having Neimann-Pick disease to treat, i.e., alleviate or reduce at least one symptom, of the disease. Symptoms of Neimann-Pick disease type A include: enlargement of the spleen and liver, lymphadenopathy, microcytic anemia, decreased platelet count, hypotonia, muscular weakness, psychomotor retardation. Symptoms of Neimann-Pick type B include: enlargement of the liver and/or spleen, hepatosplenomegaly; pulmonary compromise.

Thus, high mannose lysosomal storage enzymes such as high mannose acid ceramidase or high mannose acid sphingomyelinase can be produced by the methods described herein in order to target these proteins to mannose receptor-expressing cells.

EXAMPLES

In experiments with HT-1080 cells in which the glucocerebrosidase gene has been activated as described in U.S. 5,641,670 (Gene-Activated™ GCB (GA-GCB)), the cells were treated with either kifunensine or swainsonine at concentrations ranging from 0.1 to 2 µg/mL.

Effect of Kifunensine or Swainsonine on GA-GCB Glycoforms

HT-1080 cells producing GA-GCB were plated in duplicate 6-well plates and the Production Medium adjusted to the following concentrations of kifunensine or swainsonine: 0 (no drug), 0.1, 0.25, 0.5, 1, and 2 $\mu\text{g/mL}$. The medium was harvested and the cells refed every 24 hours for three days. The samples from the third day were subjected to isoelectric focusing (IEF) analysis. The effect of kifunensine and swainsonine on the molecular charge of GA-GCB is shown by the IEF analysis. With both drugs, a concentration dependent increase in the apparent isoelectric point (pI) was observed, with kifunensine causing a much larger shift in pI than swainsonine at the highest concentration tested (2 $\mu\text{g/mL}$).

Effect of Kifunensine or Swainsonine on GA-GCB Production

Ten roller bottles (surface area, 1700 cm^2 each) were seeded in Growth Medium (DMEM with 10% calf serum) with HT-1080 cells producing GA-GCB. Following two weeks of growth, the medium was aspirated and 200 mL of fresh Production Medium (DMEM/F12, 0% calf serum) was added to three sets of roller bottles. Two sets of 4 roller bottles were treated with 1 $\mu\text{g/mL}$ of either kifunensine or swainsonine. The third group of two roller bottles received no drug treatment. After approximately 24 hours, the medium from each roller bottle was harvested, pooled and a sample taken for GA-GCB enzymatic activity analysis. This procedure was repeated for seven days. Stable production of GA-GCB was observed for all roller bottles throughout the seven daily harvests (Table 1). Absolute levels of the enzyme, however, varied according to drug treatment group with the following average GA-GCB production levels observed across the seven harvests: 38.3 ± 3.5 mg/L (control, no drug treatment), 24.5 ± 4.0 mg/L (swainsonine, 1 $\mu\text{g/mL}$), and 21.3 ± 2.8 mg/L (kifunensine, 1 $\mu\text{g/mL}$). Both drugs, therefore, resulted in stable, but lower production levels with the largest decrease seen for kifunensine (44% reduction relative to control).

TABLE 1

Roller Bottle Production of Glucocerebrosidase in Cells Treated with Mannosidase Inhibitors								
Treatment	Glucocerebrosidase ^{a)} Activity (^{b)} mg/Liter)							Average \pm Standard Deviation
	Harvest 1	Harvest 2	Harvest 3	Harvest 4	Harvest 5	Harvest 6	Harvest 7	
No drug added	35.8	36.6	44.9	40.5	34.6	38.3	37.2	38.3 ± 3.5
Swainsonine (1 $\mu\text{g/mL}$)	28.6	17.4	28.5	27.0	22.9	25.0	22.3	24.5 ± 4.0
Kifunensine (1 $\mu\text{g/mL}$)	26.0	22.9	17.7	21.2	18.4	21.0	22.0	21.3 ± 2.8

^{a)} Assay performed as follows: test article is mixed with the enzyme substrate (4-methylumbelliferyl- β -D-glucopyranoside) and incubated for 1 hour at 37° C. The reaction is stopped by the addition of NaOH/Glycine buffer. Fluorescence is quantified by the use of a fluorescence spectrophotometer.

^{b)} Specific activity: 2,500 Units/mg. One unit is defined as the conversion of 1 μMole of substrate in 1 hour at 37° C.

Effect of Kifunensine or Swainsonine on GA-GCB Uptake into Macrophages

GA-GCB produced in HT-1080 cells was used in an in vitro assay to determine uptake efficiency in a mouse macrophage cell line. The specific objective of the experiment

was to determine the absolute and mannose receptor-specific uptake of GA-GCB in mouse J774E cells. One day prior to assay, J774E cells were plated at 50,000 cells/ cm^2 in 12 well plates in Growth Medium. For the assay, 0.5 mL of Production Medium (DMEM/F12, 0% calf serum) containing 50 nM vitamin D3 (1,2-5, Dihydroxy vitamin D3) was added to the cells. Unpurified GA-GCB (from harvest 4, Table 1) was added to the test wells at a final concentration of 10 $\mu\text{g/mL}$ in the presence or absence of 2 $\mu\text{g/mL}$ mannan (a competitor for the mannose receptor). Three different forms of GA-GCB were used: GA-GCB from cells treated with kifunensine (1 $\mu\text{g/mL}$), GA-GCB from cells treated with swainsonine (1 $\mu\text{g/mL}$), and GA-GCB (1 $\mu\text{g/mL}$) from untreated cells. Control wells received no GA-GCB. The wells were incubated for 4 hours at 37° C., then washed extensively in buffered saline, scraped into GA-GCB enzyme reaction buffer, passed through 2 freeze/thaw cycles, and clarified by centrifugation. The supernatant was then quantitatively tested for enzyme activity and total protein. Internalization of GA-GCB into mouse J774E cells is shown in Table 2 and is reported as Units/mg of cell lysate. These results demonstrated that uptake of GA-GCB from kifunensine treated cells was 14-fold over background and 73% inhibitable by mannan and that uptake of GA-GCB from swainsonine treated cells was 7-fold over background and 67% inhibitable by mannan. In addition, they also demonstrate that uptake of GA-GCB from untreated cells was approximately 3-fold over background and 53% inhibitable by mannan. Thus, the inhibition of intracellular mannosidases by either kifunensine or swainsonine results in GA-GCB that can be transported into cells efficiently via the mannose receptor, with kifunensine causing an approximately 2-fold greater uptake than swainsonine. Improvement in targeting of GA-GCB to cells via mannose receptors can therefore be optimized by production of GA-GCB in the presence of kifunensine or swainsonine.

TABLE 2

Internalization of Glucocerebrosidase Into J774E Cells.
Glucocerebrosidase Produced from Cells Treated with Mannosidase Inhibitors

^{a)} Sample	^{b)} Glucocerebrosidase Activity (Units/mg cell lysate)		
	Absolute	Background Corrected	Inhibition (%)
Background (no GA-GCB added)	655	0	—
GA-GCB from untreated cells + Mannan	2816	2161	53
GA-GCB from kifunensine treated cells + Mannan	9185	8530	73
GA-GCB from swainsonine treated cells + Mannan	4787	4132	67

^{a)} Assay performed as follows: sample is mixed with the enzyme substrate (4-methylumbelliferyl- β -D-glucopyranoside) and incubated for 1 hour at 37° C. The reaction is stopped by the addition of NaOH/Glycine buffer. Fluorescence is quantified by the use of a fluorescence spectrophotometer. Total protein determined in freezer/thaw cell lysates by bicinchoninic acid (BCA). Activity reported as units/mg total protein. One Unit is defined as the conversion of 1 μMole of substrate in 1 hour at 37° C.

^{b)} Cells treated with drug received 1 $\mu\text{g/mL}$ of either Kifunensine or Swainsonine in the presence or absence of mannan (2 $\mu\text{g/mL}$).

Purification and Characterization of hmGCB

HmGCB was purified from the culture medium of human fibroblasts grown in the presence of kifunensine at a con-

centration of 2 µg/ml. The four N-linked glycans present on hmGCB were released by peptide N-glycosidase F and purified using a Sep-pak C18 cartridge. Oligosaccharides eluting in the 5% acetic acid fraction were permethylated using sodium hydroxide and methyl iodide, dissolved in methanol:water (80:20), and portions of the permethylated glycan mixture were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF-MS). The sample was analyzed on a Voyager STR Biospectrometry Research Station laser-desorption mass spectrometer coupled with Delayed Extraction using a matrix of 2,5-dihydroxybenzoic acid. A pattern of pseudo-molecular ions is seen in the range m/z 1500–2500, indicating the presence of high-mannose glycans ranging from Man₅GlcNAc₂ to Man₉GlcNAc₂.

TABLE 3

Summary of Data Obtained from MALDI-TOF-MS Analysis of N-Glycans from hmGCB from Kifunensine-Treated Cells

M/Z	Peak Assignment	Approximate % of Total Glycans
1580	Man ₅ GlcNAc ₂	1.3
1730	Man ₆ GlcNAc ₂	11.2
1752		
1784		
1934	Man ₇ GlcNAc ₂	23.3
1957		
1988		
2139	Man ₈ GlcNAc ₂	32.0
2161		
2192		

TABLE 3-continued

Summary of Data Obtained from MALDI-TOF-MS Analysis of N-Glycans from hmGCB from Kifunensine-Treated Cells

M/Z	Peak Assignment	Approximate % of Total Glycans
2343	Man ₉ GlcNAc ₂	51.2
2365		
2397		
2969	Biantennary complex	1.0

The most abundant high mannose glycans present are Man₉GlcNAc₂ and Man₈GlcNAc₂, with decreasing abundances of Man₇GlcNAc₂, Man₆GlcNAc₂, and Man₅GlcNAc₂. A trace amount of a fucosylated biantennary complex glycan containing two sialic acid residues was observed. An approximate indication of the relative abundance of each glycan is obtained by measuring the peak heights. See Table 3. A more accurate assessment of the average chain length of the high mannose glycans was obtained by MALDI-TOF-MS analysis of the intact glycoprotein. A sharp peak was obtained at m/z 62,483.1 due to the homogeneity of the glycan chains. The mass of the mature peptide calculated from the amino acid sequence is 55,577.6, indicating the four N-linked glycan chains contribute 6905.5 to the total mass of hmGCB. From this number, it can be calculated that the average glycan length is 8.15 mannose residues.

All patents and references cited herein are incorporated in their entirety by reference.

Other embodiments are within following claims.

SEQUENCE LISTING

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<211> LENGTH: 8850

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 1

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What is claimed:

1. A method of producing a preparation of high mannose 60
 glucocerebrosidase (hmGCB) comprising a carbohydrate
 chain having at least four mannose residues, comprising:
 providing a mammalian cell that expresses a human
 glucocerebrosidase (GCB);
 contacting the cell with kifunensine;
 allowing the cell to produce hmGCB; and

harvesting the hmGCB from the cell or its culture media,
 to thereby produce an hmGCB preparation.

2. The method of claim 1, wherein removal of one or more
 α 1,2 mannose residue(s) distal to the pentasaccharide core
 is prevented.

3. The method of claim 1, wherein the kifunensine is
 65 present at a concentration between about 0.05 to 20.0 μg/ml.

4. The method of claim 3, wherein the kifunensine is
 present at a concentration between about 0.1 to 2.0 μg/ml.

5. The method of claim 1, further comprising contacting the cell with a class 2 processing mannosidase inhibitor.

6. The method of claim 5, wherein the class 2 processing mannosidase inhibitor is selected from the group consisting of: swainsonine, mannostatin, 6-deoxy DIM, 6-deoxy-6-fluoro-DIM and combinations thereof.

7. The method of claim 5, wherein the class 2 processing mannosidase inhibitor is swainsonine.

8. The method of claim 5, wherein the class 2 processing mannosidase inhibitor is present at a concentration between 0.05 to 20.0 $\mu\text{g/ml}$.

9. The method of claim 1, wherein the hmGCB has at least one carbohydrate chain having five mannose residues.

10. The method of claim 1, wherein the hmGCB has at least one carbohydrate chain having eight mannose residues.

11. The method of claim 1, wherein the hmGCB has at least one carbohydrate chain having nine mannose residues.

12. The method of claim 1, wherein the removal of one or more mannose residues distal to the pentasaccharide core is prevented on at least two carbohydrate chains of hmGCB.

13. The method of claim 1, wherein at least 60% of the hmGCB of the preparation have one or more carbohydrate chains in which the removal of one or more mannose residues distal to the pentasaccharide core has been prevented.

14. The method of claim 13, wherein the removal of three or more mannose residues distal to the pentasaccharide core has been prevented.

15. The method of claim 1, wherein at least about 20% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.

16. The method of claim 15, wherein at least about 40% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.

17. The method of claim 16, wherein at least about 60% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.

18. The method of claim 1, wherein at least about 80% or more of the carbohydrate chains of the hmGCB preparation have six or more mannose residues.

19. The method of claim 1, wherein the cell is a human cell and is a knockout for a class 2 processing mannosidase.

20. The method of claim 1, wherein the cell is a human cell and comprises a class 2 processing mannosidase antisense molecule.

21. The method of claim 1, wherein the cell comprises an exogenous nucleic acid sequence comprising a GCB coding region.

22. The method of claim 21, wherein the cell further comprises an exogenous regulatory sequence which functions to regulate expression of the GCB coding region.

23. The method of claim 1, wherein the cell comprises an exogenous regulatory sequence which functions to regulate expression of an endogenous GCB coding sequence.

24. The method of claim 1, wherein the cell is a primary cell.

25. The method of claim 1, wherein the cell is a secondary cell.

26. The method of claim 1, wherein the cell is a human cell.

27. The method of claim 26, wherein the cell is a fibroblast or a myoblast.

28. The method of claim 26, wherein the cell is an immortalized cell.

29. The method of claim 27, wherein the cell is an HT-1080 cell.

30. The method of claim 1, wherein the cell is contacted with kifunensine in culture media.

31. The method of claim 30, wherein the hmGCB is obtained from the media in which the cell is cultured.

32. A method of producing a preparation of high mannose glucocerebrosidase (hmGCB) comprising a carbohydrate chain having at least four mannose residues, the method comprising:

providing a human cell into which a nucleic acid sequence comprising an exogenous regulatory sequence has been introduced such that the regulatory sequence is operably linked to, and regulates the expression of, an endogenous GCB coding region;

contacting the cell with a class 1 mannosidase inhibitor such that the removal of at least one mannose residue distal to the pentasaccharide core of a precursor oligosaccharide of GCB is prevented; and

allowing the cell to produce hmGCB, to thereby produce an hmGCB preparation.

33. The method of claim 32, wherein the mannosidase inhibitor prevents the removal of one or more α 1,2 mannose residue(s) distal to the pentasaccharide core.

34. The method of claim 32, wherein the mannosidase inhibitor further prevents the removal of one α 1,3 mannose residue distal to the pentasaccharide core.

35. The method of claim 32, wherein the mannosidase inhibitor further prevents the removal of one α 1,6 mannose residue distal to the pentasaccharide core.

36. The method of claim 32, wherein the class 1 processing mannosidase inhibitor is kifunensine.

37. The method of claim 36, wherein the kifunensine is present at a concentration between about 0.05 to 20.0 $\mu\text{g/ml}$.

38. The method of claim 37, wherein the kifunensine is present at a concentration between about 0.1 to 2.0 $\mu\text{g/ml}$.

39. The method of claim 32, wherein the cell is further contacted with a class 2 mannosidase inhibitor.

40. The method of claim 39, wherein the class 2 processing mannosidase inhibitor is selected from the group consisting of: swainsonine, mannostatin, 6-deoxy DIM, 6-deoxy-6-fluoro-DIM and combinations thereof.

41. The method of claim 39, wherein the class 2 processing mannosidase inhibitor is swainsonine.

42. The method of claim 39, wherein the class 2 processing mannosidase inhibitor is present at a concentration between 0.05 to 20.0 $\mu\text{g/ml}$.

43. The method of claim 32, wherein the cell is a knockout for a class 2 processing mannosidase.

44. The method of claim 32, wherein the cell comprises a class 2 processing mannosidase antisense molecule.

45. The method of claim 32, wherein the hmGCB has at least one carbohydrate chain having six mannose residues of the precursor oligosaccharide.

46. The method of claim 32, wherein the hmGCB has at least one carbohydrate chain having eight mannose residues of the precursor oligosaccharide.

47. The method of claim 32, wherein the hmGCB has at least one carbohydrate chain having nine mannose residues of the precursor oligosaccharide.

48. The method of claim 32, wherein the mannosidase inhibitor prevents removal of at least three mannose residues distal to the pentasaccharide core of the precursor oligosaccharide of GCB.

49. The method of claim 32, wherein the mannosidase inhibitor prevents removal of one or more mannose residues distal to the pentasaccharide core on at least two of the carbohydrate chains of hmGCB.

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50. The method of claim 32, wherein at least 60% of the hmGCB of the preparation have one or more carbohydrate chains in which the removal of three or more mannose residues distal to the pentasaccharide core has been prevented.

51. The method of claim 32, wherein at least 20% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.

52. The method of claim 51, wherein at least 40% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.

53. The method of claim 52, wherein at least 60% of the hmGCB of the preparation have one or more carbohydrate chains having at least eight mannose residues.

54. The method of claim 32, wherein at least about 80% or more of the carbohydrate chains of the hmGCB preparation have six or more mannose residues.

55. The method of claim 32, wherein the cell is a primary cell.

56. The method of claim 32, wherein the cell is a secondary cell.

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57. The method of claim 32, wherein the cell is a fibroblast or a myoblast.

58. The method of claim 32, wherein the cell is an immortalized cell.

59. The method of claim 58, wherein the cell is an HT-1080 cell.

60. The method of claim 36, wherein the cell is contacted with kifunensine in culture media.

61. The method of claim 60, wherein the hmGCB is obtained from the media in which the cell is cultured.

62. The method of claim 1, wherein the cell is a Chinese hamster ovary (CHO) cell transfected with an exogenous nucleic acid sequence comprising a human GCB coding sequence.

63. The method of claim 1, wherein the cell is a COS cell transfected with an exogenous nucleic acid sequence comprising a human GCB coding sequence.

* * * * *

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment E

Maintenance Fee Statement

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PATENT NUMBER	FEE AMT	SUR CHARGE	PYMT DATE	U.S. APPLICATION NUMBER	PATENT ISSUE DATE	APPL. FILING DATE	PAYMENT YEAR	SMALL ENTITY?	ATTY DKT NUMBER
7,138,262	\$980.00	\$0.00	03/25/10	09/641,471	11/21/06	08/18/00	04	NO	HGT 0013

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment F

Brumshtein et al. (2010) Glycobiology 20(1):24-32

Characterization of gene-activated human acid- β -glucosidase: Crystal structure, glycan composition, and internalization into macrophages

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Received on June 1, 2009; revised on August 30, 2009; accepted on September 1, 2009

Gaucher disease, the most common lysosomal storage disease, can be treated with enzyme replacement therapy (ERT), in which defective acid- β -glucosidase (GlcCerase) is supplemented by a recombinant, active enzyme. The X-ray structures of recombinant GlcCerase produced in Chinese hamster ovary cells (imiglucerase, Cerezyme[®]) and in transgenic carrot cells (prGCD) have been previously solved. We now describe the structure and characteristics of a novel form of GlcCerase under investigation for the treatment of Gaucher disease, Gene-ActivatedTM human GlcCerase (velaglucerase alfa). In contrast to imiglucerase and prGCD, velaglucerase alfa contains the native human enzyme sequence. All three GlcCerasees consist of three domains, with the active site located in domain III. The distances between the carboxylic oxygens of the catalytic residues, E340 and E235, are consistent with distances proposed for acid–base hydrolysis. Kinetic parameters (K_m and V_{max}) of velaglucerase alfa and imiglucerase, as well as their specific activities, are similar. However, analysis of glycosylation patterns shows that velaglucerase alfa displays distinctly different structures from imiglucerase and prGCD. The predominant glycan on velaglucerase alfa is a high-mannose type, with nine mannose units, while imiglucerase contains a chitobiose tri-mannosyl core glycan with fucosylation. These differences in glycosylation affect cellular internalization; the rate of velaglucerase alfa internalization into human macrophages is at least 2-fold greater than that of imiglucerase.

Keywords: Gaucher disease/gene activation/
glucocerebrosidase/glycans/mannose-6-phosphate receptor/
site-specific glycosylation/X-ray structure

Introduction

Gaucher disease is caused by mutations in the gene encoding the lysosomal enzyme, acid- β -glucosidase (glucocerebrosidase, GlcCerase, E.C. 3.2.1.45) (Beutler and Grabowski 2001;

Futerman and Zimran 2006). The most common treatment for Gaucher disease is enzyme replacement therapy (ERT), in which defective GlcCerase is supplemented with an active enzyme. ERT using imiglucerase, a recombinant analog of human GlcCerase expressed in Chinese hamster ovary (CHO) cells has been available for ~15 years. After expression and purification, imiglucerase is modified by exo-glycosidase treatment (Friedman and Hayes 1996) to expose the core mannose residues that can be recognized by macrophages. Glycan remodeling greatly improves targeting to and internalization by macrophages, the main cell type affected in Gaucher disease (Futerman and Zimran 2006). An alternative means of producing GlcCerase (prGCD) in transgenic carrot root cells has been developed (Aviezer et al. 2009). The X-ray structures of imiglucerase and prGCD have been previously reported (Dvir et al. 2003; Shaaltiel et al. 2007).

In the current study, we have used gene activation in a well-characterized, continuous human cell line to produce gene-activated human acid- β -glucocerebrosidase (velaglucerase alfa). Gene activation refers to targeted recombination with a promoter that activates the endogenous GlcCerase gene in the selected human cell line. Velaglucerase alfa is secreted as a monomeric glycoprotein of approximately 63 kDa and is composed of 497 amino acids with a sequence identical to that of the natural human protein (Zimran et al. 2007). Glycosylation of velaglucerase alfa is altered by using kifunensine, a mannosidase I inhibitor, during cell culture, which results in the secretion of a protein containing predominantly high-mannose type glycans (Elbein et al. 1990).

Herein we describe the crystal structure of velaglucerase alfa, using a preparation that had been partially deglycosylated, and show that it is similar to that of imiglucerase (Dvir et al. 2003) and prGCD (Shaaltiel et al. 2007). Velaglucerase alfa differs from imiglucerase and prGCD as the latter two enzymes contain a mutation at residue 495 (an Arg to His substitution: R495H), and prGCD contains seven additional residues at the C terminus (DLLVDTM) and two additional residues at the N terminus (EF). Moreover, the kinetic parameters and specific activity of velaglucerase alfa are very similar to those of imiglucerase. We also compare the glycosylation patterns of velaglucerase alfa and imiglucerase by use of LC-MS and assess the impact of the different glycosylation patterns by analyzing internalization in human macrophages.

Results and discussion

X-ray structure

Diffraction-quality crystals of velaglucerase alfa were obtained after partial deglycosylation using *N*-glycosidase F, by a procedure similar to that previously described for imiglucerase (Dvir et al. 2003). Velaglucerase alfa crystallized in the same space

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Table I. Data collection and refinement statistics

	Velaglucerase alfa
Data collection	
Space group	C222 ₁
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	109.37, 285.55, 91.69
<i>abg</i> (°)	90.00, 90.00, 90.00
Resolution (Å)	19.9–2.7 (2.75–2.70) ^a
<i>R</i> _{sym} (%)	15.7 (51.0)
<i>I</i> / <i>σ</i> (<i>I</i>)	14.4 (4.6)
Completeness (%)	100 (100)
Redundancy	7.5 (7.6)
Refinement	
Resolution (Å)	19.9–2.7
Number of reflections	39,776
<i>R</i> _{work} / <i>R</i> _{free}	17.3/23.4
Rms deviations	
Bond lengths (Å)	0.012
Bond angles (°)	1.486
Number of refined atoms	
Protein	7871
Carbohydrates	70
Ions	90
Solvent	326
Ramachandran outliers (%)	0.4

^aThe highest resolution shell is shown in parentheses.

group, C222₁, as imiglucerase (Table I), and unit cell parameters were similar to the previously published GlcCerase structures (Dvir et al. 2003; Premkumar et al. 2005; Brumshtein et al. 2006). The asymmetric unit contained two copies of velaglucerase alfa, designated as molecules A and B. The root mean square deviation (RMSD) value between molecules A and

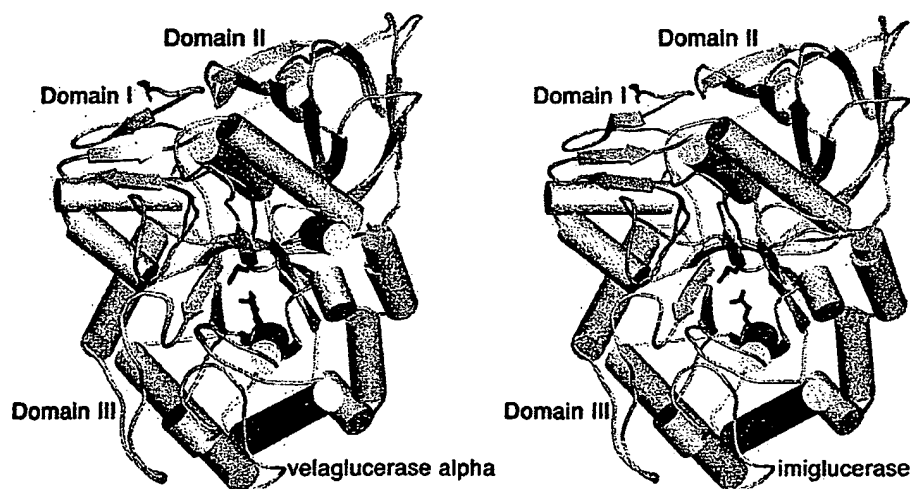
B (<0.3 Å) shows that they are virtually identical. A comparison of the structures of imiglucerase, prGCD, and velaglucerase alfa demonstrates that these structures are very similar, with an RMSD of 0.35–0.46 Å (Table II).

Velaglucerase alfa thus consists of three noncontiguous domains, with the catalytic site located in domain III (residues 76–381 and 416–430), which is a (β/α)₈ (TIM) barrel (Figure 1). A more detailed analysis of the active site reveals that it is virtually identical to that of imiglucerase (Figure 2), with the distances between the carboxylic oxygens of the catalytic residues, E340 and E235 (5.2 Å in molecule A and 5.1 Å in molecule B), similar to those obtained previously (Brumshtein et al. 2006) and in agreement with the distances proposed for acid–base hydrolysis (Davies and Henrissat 1995). Moreover, the three loops (loop 1, residues 345–350; loop 2, residues 393–399; and loop 3, residues 312–319) observed in previous structures (reviewed in Kacher et al. (2008)) are also seen in velaglucerase alfa. Similarly to prGCD (Shaaltiel et al. 2007), loops 2 and 3 show differences in their backbone angles and side chain orientations in the two molecules of the asymmetric unit, whereas loop 1, since it makes crystal contacts, exhibits less pronounced conformational changes (Figure 2). In the case of loop 3, a helical conformation is seen in molecule B, whereas a coiled conformation is seen in molecule A (Figure 3), as previously reported for imiglucerase (Brumshtein et al. 2006). Although the crystal was cryo-protected with 25% ethylene glycol, we did not detect any ethylene glycol molecules in the electron density map.

Imiglucerase and prGCD both contain an Arg to His mutation at residue 495, with H495 making an H-bond (2.6 Å) with the peptide carbonyl of F31. In contrast, velaglucerase alfa contains a sequence identical to that of the natural human enzyme,

Table II. RMS deviations of velaglucerase alfa compared to imiglucerase and prGCD. RMS deviations (Å) are shown for each of the two copies of the molecules in the asymmetric unit and were calculated using PyMol (www.pymol.org). The PDB codes for imiglucerase and pr-GlcCerase are 2J25 and 2V3F, respectively

	Imiglucerase-A	Imiglucerase-B	prGCD-A	prGCD-B
Velaglucerase alfa-A	0.39	0.35	0.36	0.40
Velaglucerase alfa-B	0.38	0.43	0.46	0.46

**Fig. 1.** Comparison of the crystal structures of velaglucerase alfa and imiglucerase. The three domains of the enzymes are colored pink (domain I, residues 1–29 and 383–414), blue (domain II, residues 30–75 and 431–497), and gray (domain III, residues 76–382 and 415–430).

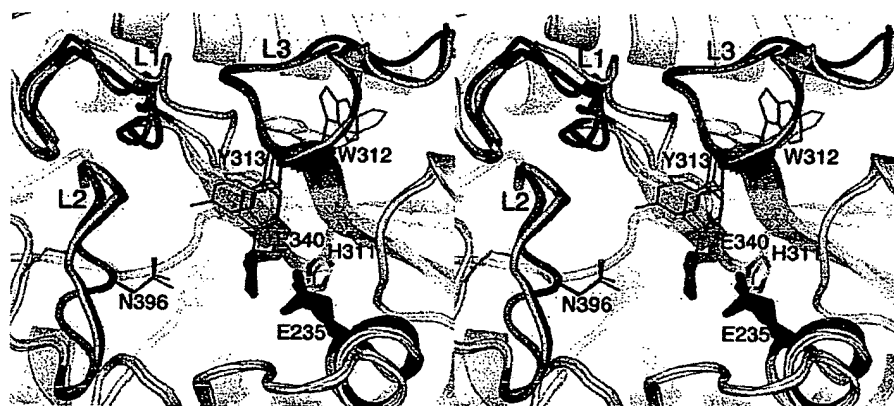


Fig. 2. Active site of velaglucerase alfa. Stereo representation of an overlay of the active sites of imiglucerase (blue and magenta) and velaglucerase alfa (yellow and green). Catalytic residues are shown as red sticks. Loops near the entrance to the active site are indicated (L1, loop 1; L2, loop 2; L3, loop 3).

with an Arg at residue 495, which does not make a similar H-bond. No major structural differences were observed in velaglucerase alfa around residue R495, relative to imiglucerase or prGCD. Two mutations which cause Gaucher disease, R496 and D474 (Figure 4) (Kawame et al. 1992; Beutler et al. 1993; Choy et al. 1998), are in close proximity to R495 near the N-terminus of GlcCer. D474 is at the end of a β -strand, and R496 is part of a coil with no clear secondary structure, and their side-chains form a salt bridge and hydrogen bonds with each other; mutations in either of these two residues would disrupt these interactions. By analyzing the geometry and the interactions between the side chains of these two residues, and the secondary structure of the region, we conclude that R496 or D474 may be involved in stabilizing the conformation of the N-terminus of the enzyme by their side chain interactions, with disruption of these bonds resulting in a flexible N-terminus and hence in a less stable structure. However, neither of these residues interacts with R495.

Kinetic analysis

To further compare velaglucerase alfa and imiglucerase, and to determine if the mutation at residue 495 has any effect, kinetic parameters and specific activity were determined using a natural glucosylceramide (GlcCer) substrate, rather than a surrogate substrate typically used to assess enzyme activity. Velaglucerase alfa has a k_{cat} of 2100 min^{-1} , a K_m of $19 \mu\text{M}$, and a V_{max} of $0.61 \mu\text{M min}^{-1}$. Imiglucerase has a k_{cat} of 1900 min^{-1} , a K_m of $15 \mu\text{M}$, and a V_{max} of $0.56 \mu\text{M min}^{-1}$ (Figure 5). Similar K_m values were reported in the literature; GlcCer derived from brain tissue and fibroblasts both have a K_m of $32 \mu\text{M}$ using GlcCer from Gaucher spleen (Vaccaro et al. 1982), while imiglucerase and prGCD have a K_m of 15.2 and $20.7 \mu\text{M}$, respectively, using a fluorescent GlcCer analog, C6-NBD-GlcCer (Shaaltiel et al. 2007). In addition, at a $210 \mu\text{M}$ GlcCer substrate concentration, velaglucerase alfa and imiglucerase have similar specific activities of 26 and 24 U/mg , respectively. These results

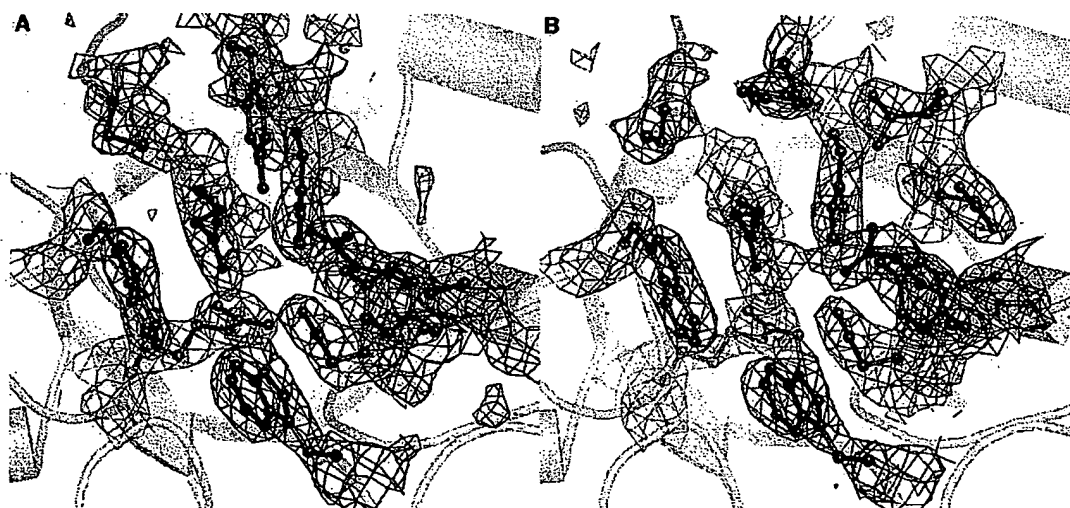


Fig. 3. Electron density around the catalytic center. Catalytic residues E235 and E340 are shown as red balls and sticks and surrounding residues are in dark gray. Contours of the $2F_o - F_c$ map are shown as a blue mesh (at 1.2σ); contours of the $F_o - F_c$ map are shown in green mesh (at 3σ) and in magenta (at -3σ). Several $F_o - F_c$ peaks are visible in the active site, but they did not overlap with the $2F_o - F_c$ map, nor are they continuous; hence, at this resolution they appear to be noise. A and B show the catalytic centers of molecules A and B, respectively, in the asymmetric unit.

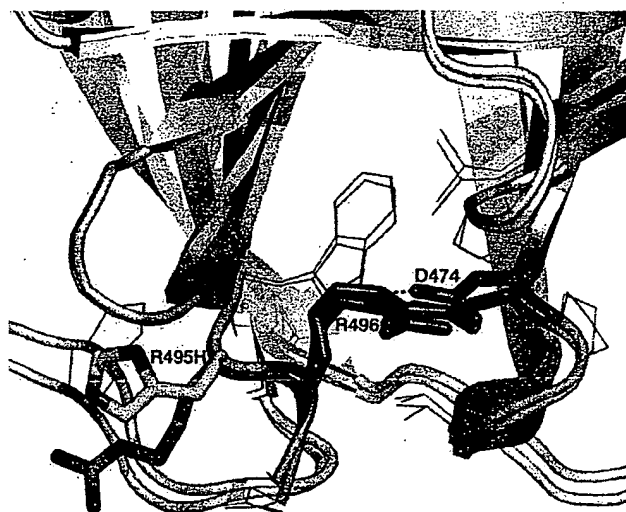


Fig. 4. Mutations at the C-terminus of GlcCerase. Imiglucerase and pr-GlcCerase contain a His at residue 495 (yellow), whereas velaglucerase alfa contains Arg (green). Mutations R496 and D474, which cause Gaucher disease, are shown in magenta. Residues within 4 Å distance of R495 and R496 are shown in cyan.

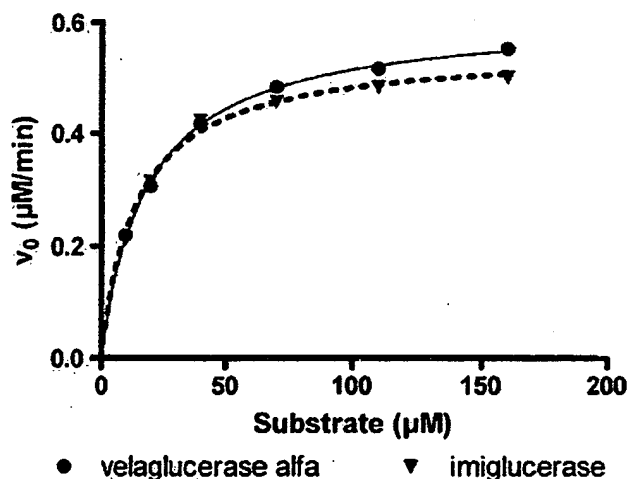


Fig. 5. Kinetic analysis of velaglucerase alfa and imiglucerase. V_{max} and K_m values were determined using a natural GlcCer substrate ($n = 2$).

demonstrate that human and CHO-cell derived GlcCerase, prepared by two different manufacturing processes, have similar enzymatic activities for the natural substrate.

Glycan composition

We next examined which sugars could be detected in the crystal structure of velaglucerase alfa. Even after partial deglycosylation using *N*-glycosidase F, two sugar residues were observed attached to residue N19 in both molecules A and B (Figure 6). One sugar was detected on N146 in molecule A whereas no sugars were detected on N146 in molecule B (Figure 6). As reported previously for imiglucerase, no sugars were detected attached to either N270 or N59 in velaglucerase alfa. It should

be noted that sugars attached to N270 have not been seen in any of the crystal structures solved to date, and sugars have been seen only occasionally on N59 (Brumshtein et al. 2006). The inability to detect sugars on either N59 or N270 is most likely due to the high flexibility of the corresponding glycan chains since nano-liquid chromatography electrospray ionization tandem mass spectrometry (nano-LC-ESI-MS/MS) analysis of intact imiglucerase (Kacher et al. 2008), and of velaglucerase alfa (see below) showed that glycan chains were attached to both these residues.

Velaglucerase alfa and imiglucerase bear distinctly different glycan chains due to the differences in their manufacture. In our comparative study of the carbohydrate content of unmodified velaglucerase alfa and imiglucerase by LC-ESI-MS, four of the five potential glycosylation sites, namely, N19, N59, N146, and N270, were observed to be fully occupied in both. As expected from the crystal structures, N462 is fully unoccupied in both, due to its buried location.

According to LC-ESI-MS analysis of glycopeptide maps, velaglucerase alfa contains primarily high-mannose type glycans, consisting of six to nine mannose units. Listed as the predominant structure in Table III, the most abundant ion present in the averaged spectra for each site corresponds to a glycan with nine mannose units. Glycan microheterogeneity was observed at each site and the less abundant structures are listed as other glycans. These other glycans consist of mannose residues with phosphorylation at the C-6 position to create a mannose-6-phosphate (M6P) residue. The lowest levels of M6P were at N19; N59 and N146 had similar but higher levels relative to N19, while N270 had the highest amount of M6P. Despite the site-specific variation in relative levels of M6P, nonphosphorylated glycans remained the predominant species for all four sites. Also observed on N59, N146 and N270 were mono-sialylated mono-antennary hybrid and complex-type structures with core fucosylation, which were quantified by glycan map analysis. These structures are consistent with a low percentage of glycosylation sites escaping kifunensine inhibition, resulting in glycan maturation and core fucosylation. In the case of hybrid-type glycans, only a single antenna matured.

The results from site-specific glycan characterization were corroborated by glycan map analysis (Figure 8), which demonstrates high-mannose type glycans consisting of six to nine mannose units with a predominant nine-mannose structure. Estimates from glycan map analysis show that the mono-sialylated mono-antennary hybrid structures account for ~2% of the total glycan pool. The map also demonstrates the presence of high-mannose glycans containing one GlcNAc-capped M6P, a result of incomplete glycan processing, as well as high-mannose glycans bearing a single M6P. Also consistent with these results were data obtained from monosaccharide compositional analysis that demonstrates approximately 0.8 mole of M6P per mole of velaglucerase alfa, and approximately 0.6 moles of M6P per mole of imiglucerase.

Site-specific glycan analysis demonstrated that imiglucerase contains primarily complex-type glycans with core fucosylation that terminate with the chitobiose tri-mannosyl core (Table IV), with an exception at the N19 site, which was observed to be devoid of fucose. These structures are as expected for GlcCerase with exoglycosidase treatment to expose the core mannose residues. Imiglucerase also contains glycan microheterogeneity at each site of glycosylation, with lower

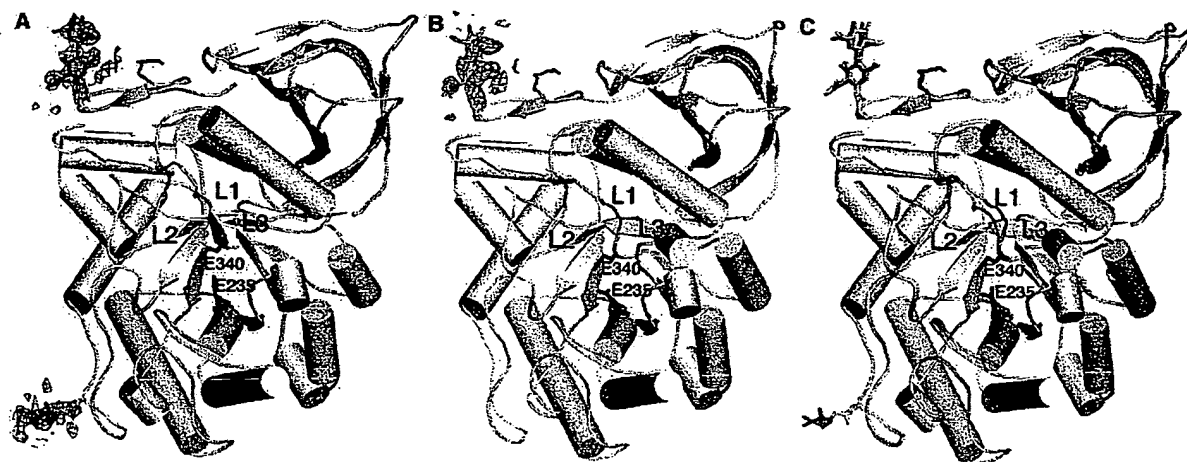


Fig. 6. Glycosylation sites seen in the crystal structure of velaglucerase alfa. $2F_o - F_c$ electron density maps are shown, which are contoured at 1.2σ in the vicinity of two of the putative glycosylation sites, N19 and N146 for molecule A, and N19 for molecule B. (A) Glycosylation sites detected in molecule A are shown in green. (B) Glycosylation site detected in molecule B is shown in yellow. (C) Superposition of the two individual molecules in the asymmetric unit reveals their similarity. In all three representations, catalytic residues E235 and E340 are shown as red sticks.

Table III. Carbohydrate composition of velaglucerase alfa. Predominant structures are those observed to be most abundant at each *N*-linked glycosylation site. The other glycans consisted mostly of high-mannose type structures (some with M6P) and with the hybrid and complex types observed at low levels ($\sim 2\%$ of the total as determined by glycan map analysis)

Glycosylation site	Predominant glycan	Other glycans
Asn19	High mannose (Man) ₉ (GlcNAc) ₂	High mannose (Man) ₆₋₈ (GlcNAc) ₂ Phosphorylated high mannose (Phos) ₁ (Man) ₈₋₉ (GlcNAc) ₂ GlcNAc-capped phosphate (Phos) ₁ (Man) ₈₋₉ (GlcNAc) ₃ Hybrid (Hex) ₂ (Man) ₃ (GlcNAc) ₃ (Fuc) ₁
Asn59	High mannose (Man) ₉ (GlcNAc) ₂	High mannose (Man) ₅₋₈ (GlcNAc) ₂ Phosphorylated high mannose (Phos) ₁ (Man) ₇₋₉ (GlcNAc) ₂ GlcNAc-capped phosphate (Phos) ₁ (Man) ₈₋₉ (GlcNAc) ₃ Hybrid (NeuAc) ₁ (Gal) ₁ (Man) ₅ (GlcNAc) ₃ (Fuc) ₁ Complex (NeuAc) ₀₋₂ (Gal) ₂ (Man) ₃ (GlcNAc) ₄ (Fuc) ₁ (Gal) ₃ (Man) ₃ (GlcNAc) ₅ (Fuc) ₁
Asn146	High mannose (Man) ₉ (GlcNAc) ₂	High mannose (Man) ₆₋₈ (GlcNAc) ₂ Phosphorylated high mannose (Phos) ₁ (Man) ₇₋₉ (GlcNAc) ₂ GlcNAc-capped phosphate (Phos) ₁ (Man) ₉ (GlcNAc) ₃ Hybrid (NeuAc) ₁ (Gal) ₁ (Man) ₅ (GlcNAc) ₃ (Fuc) ₁
Asn270	High mannose (Man) ₉ (GlcNAc) ₂	High mannose (Man) ₆₋₈ (GlcNAc) ₂ Phosphorylated high mannose (Phos) ₁ (Man) ₆₋₉ (GlcNAc) ₂ GlcNAc-capped phosphate (Phos) ₁ (Man) ₉ (GlcNAc) ₃ Hybrid (Gal) ₁ (Man) ₇ (GlcNAc) ₃ (Fuc) ₁ (NeuAc) ₁ (Gal) ₁ (Man) ₅ (GlcNAc) ₃ (Fuc) ₁ Complex (NeuAc) ₂ (Gal) ₂ (Man) ₃ (GlcNAc) ₄ (Fuc) ₁
Asn462	Not detected	Not detected

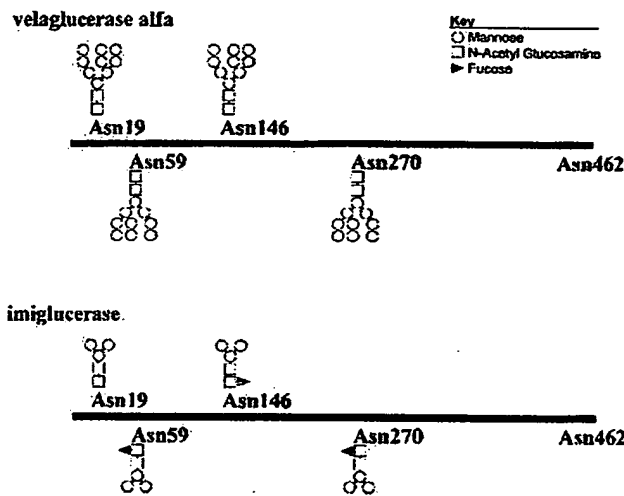


Fig. 7. Glycan structures of velaglucoerose alfa and imiglucoerose. Predominant *N*-linked carbohydrate structures on velaglucoerose alfa (top) and imiglucoerose (bottom) are shown graphically at their relative positions along the protein backbone.

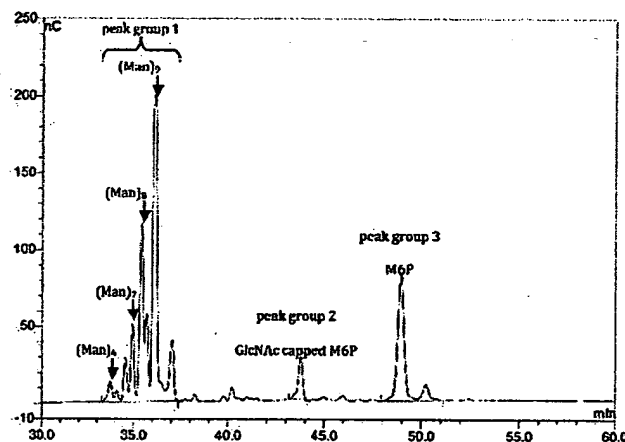


Fig. 8. Glycan map analysis of velaglucoerose alfa. Glycans released by *N*-glycosidase F were analyzed by anion-exchange chromatography with amperometric detection. The method resolves glycans based on negative charge where peak group 1 corresponds to high-mannose type neutral glycans that are resolved into multiple peaks according to the number of mannose units, peak group 2 corresponds to high-mannose type glycans with one M6P that retained its GlcNAc cap (one negative charge), and peak group 3 corresponds to high-mannose type glycans containing one fully processed M6P (two negative charges). In peak group 1, smaller peaks are resolved that correspond to positional isomers of the various oligomannose types observed.

levels of core structures terminating with *N*-acetylglucosamine (GlcNAc) that are likely a result of incomplete digestion with *N*-acetylglucosaminidase. At N146 and N270, high-mannose type glycans were observed containing five to six mannose units with one M6P.

The glycan graphics shown in Figure 7 help to visualize the predominant structures for both forms of GlcCer as described in Tables III and IV. These structures were consistent with glycan types and levels observed with glycan map analysis as well as with previous reports (Van Patten et al. 2007). In the current

Table IV. Carbohydrate composition of imiglucoerose. Predominant structures are those observed to be most abundant at each *N*-linked glycosylation site. The other glycans consisted mostly of core structures with additional GlcNAc and high-mannose structures with M6P

Glycosylation site	Predominant glycan	Other glycans
Asn19	Complex (Man) ₃ (GlcNAc) ₂	Complex (Man) ₃ (GlcNAc) ₃
Asn59	Complex (Man) ₃ (GlcNAc) ₂ (Fuc) ₁	Complex (Man) ₃ (GlcNAc) ₃ (Fuc) ₁
Asn146	Complex (Man) ₃ (GlcNAc) ₂ (Fuc) ₁	Complex (Man) ₃ (GlcNAc) ₃₋₄ (Fuc) ₁ Phosphorylated high mannose (Phos) ₁ (Man) ₅₋₆ (GlcNAc) ₂ GlcNAc-capped phosphate (Phos) ₁ (Man) ₅₋₆ (GlcNAc) ₃
Asn270	Complex (Man) ₃ (GlcNAc) ₂ (Fuc) ₁	Complex (Man) ₃ (GlcNAc) ₃₋₄ (Fuc) ₁ Phosphorylated high -mannose (Phos) ₁ (Man) ₅₋₆ (GlcNAc) ₂ GlcNAc-capped phosphate (Phos) ₁ (Man) ₅₋₆ (GlcNAc) ₃

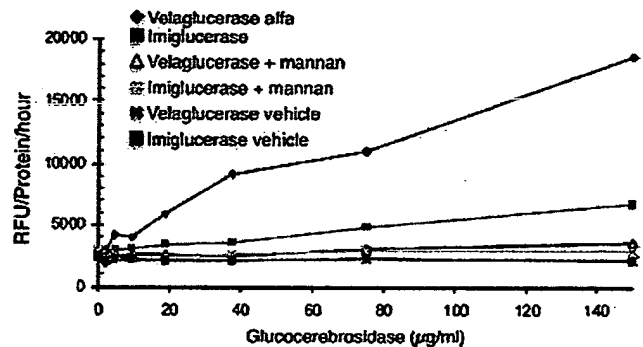


Fig. 9. Velaglucoerose alfa and imiglucoerose internalization into differentiated macrophages. The ordinate of the graph represents the fluorescence data normalized for the cellular protein concentration and incubation time (RFU/ μ g/h). The GlcCer dose is shown on the abscissa.

study, the glycans of prGCD were not characterized, but earlier studies demonstrated the presence of core α -(1,2)-xylose and core α -(1,3)-fucose (Shaaltiel et al. 2007), which are unique to plant-derived proteins and would not be expected to be present on either velaglucoerose alfa or imiglucoerose.

Internalization by macrophages

Internalization of proteins by endocytosis is highly dependent upon their carbohydrate composition and has been well characterized (Kornfeld 1986). A comparison of the internalization rate of velaglucoerose alfa to that of imiglucoerose in U937-derived macrophages demonstrated that velaglucoerose alfa is internalized approximately 2.5-fold more efficiently than imiglucoerose (Figure 9). Internalization of both enzymes could be inhibited by the addition of mannan to the culture medium, demonstrating that internalization was mediated via mannose receptors; moreover, U937 cells were shown by immunohistochemistry to express mannose receptors (CD206) (data not shown). It should be noted that during optimization of this assay, variations in results were obtained when different culture media were used.

Therefore, additional research will be required to determine the exact nature of the uptake since different mannose receptors exist, which may be involved in this cellular internalization. In contrast, the addition of M6P to the culture medium had no effect, confirming that the M6P receptor is not involved in internalization (data not shown). Since velaglycerase alfa and imiglycerase display similar kinetic parameters, specific activities, and structural features, the different rates of internalization can be ascribed to differences in glycosylation patterns between velaglycerase alfa and imiglycerase, with the increased rate of internalization of velaglycerase alfa likely due to the expression of longer chain high-mannose type glycans compared to the core mannose structures found on imiglycerase.

Conclusions

In summary, the X-ray structure of velaglycerase alfa is very similar to those of recombinant GlcCerases produced in other expression systems, with the R495H mutations found in imiglycerase and prGCD having no effect on the secondary structure. The main difference between imiglycerase and velaglycerase alfa concerns their glycan structures, with the latter containing longer chain high-mannose type glycans compared to the core mannose structures found on imiglycerase. This difference in glycosylation appears to lead to the increased cellular uptake of velaglycerase alfa over imiglycerase. The role of protein glycosylation in cellular uptake is widely established in many cell types (Barton et al. 1991). However, while the function of the macrophage mannose receptor (MR; CD206) in internalization of mannosylated proteins is well characterized (East and Isacke 2002), a growing family of carbohydrate-binding receptors have been implicated in diverse macrophage functions including removal and disposal of endotoxin (Ono et al. 2006), utilization of secreted lysosomal enzymes (Abe et al. 2008), phagocytosis (Kang et al. 2005), and regulation of the innate immune response to microbial pathogen-associated structures (Garner et al. 1994). Thus, the differences in uptake observed between imiglycerase and velaglycerase-alfa can be attributed to differences in affinity for CD206, or alternatively could be due to differential uptake mediated by other macrophage mannose receptors such as Endo180. This observed increase in cellular uptake of velaglycerase-alfa over imiglycerase can be envisioned to lead to a more rapid time to improvement of clinical parameters and potentially increased therapeutic efficacy.

Material and methods

Crystallization, structure determination, and refinement

Velaglycerase alfa was partially deglycosylated (Kacher et al. 2008) prior to crystallization, as previously described for imiglycerase (Dvir et al. 2003; Premkumar et al. 2005), using *N*-glycosidase F (88 h at 25°C), which removes carbohydrate chains from proteins and peptides by cleaving the amide bonds between Asn residues and *N*-acetylglucosamine (GlcNAc) (Han and Martinage 1992), but does not necessarily remove all carbohydrate chains from native proteins. Subsequent to *N*-glycosidase F-treatment, velaglycerase alfa was diluted in the crystallization buffer (10 mM citrate pH 5.5, 7% (v/v) ethanol, 0.02% (w/v) Na₃N₃) and passed through a Centricon YM-30 centrifugal filter device with a molecular mass cut-off of ~30 kDa, to give a final concentration of 4–5 mg/mL. Ve-

laglycerase alfa crystals were obtained by micro-batch crystallization under oil (Chayen et al. 1990) using a Douglas Instruments Oryx6 robot. The crystallization solution had a 1:1 ratio of the concentrated enzyme solution and of 1 M (NH₄)₂SO₄/0.1 M HEPES, pH 7.0, containing 0.5% (w/v) PEG8000. Crystallization was performed under Al's oil (D'Arcy et al. 1996) (1:1 ratio of paraffin and silicone liquid oils) for 5–14 days at 20°C. Data were collected on beam line ID14eh2 at the ESRF synchrotron (Grenoble, France). Crystals were cryo-protected with a 25% ethylene glycol solution, mounted, and flash cooled to 100 K. X-ray diffraction images were processed using HKL2000 and scaled with SCALEPACK (Otwinowski et al. 1997). The structure was solved using the molecular replacement method based on PDB 2J25 (Brumshtein et al. 2006) and refined with Refmac5 (Murshudov et al. 1997). During the course of refinement, the electron density map showed significant improvement, and putative sugars could be seen adjacent to N19 and N146 for molecule A, and adjacent to N19 for molecule B. Table I summarizes data collection and processing. Structures and structure factors were deposited in the PDB (code 2WKL).

Enzyme kinetics and specific activity

The novel enzymatic activity assay described below measures the ability of GlcCerase to release glucose from GlcCer obtained from Gaucher spleen (Matreya LLC, PA, Cat. no. 1057). Velaglycerase alfa (drug substance lot EP06-003) and imiglycerase (commercial product lot C7036C01) were assayed. The released glucose was quantified by anion-exchange chromatography equipped with a pulsed amperometric detector. The appropriate amount of GlcCer in chloroform/methanol (2:1, v/v) was dried by a SpeedVac in the presence of 0.2 M taurocholic acid in methanol and 20% (v/v) oleic acid in chloroform/methanol (2:1). The dried pellet was reconstituted in the 0.1 M citrate/0.2 M phosphate buffer (pH 5.0) and diluted to the desired concentrations. Enzyme samples were diluted to a concentration of 0.2 ng/μL with the dilution buffer (50 mM sodium citrate, pH 6.0 with 0.75 mg/mL BSA) and 2 ng of enzyme was incubated for 30 min at 37°C with serial dilutions of GlcCer in a 110 μL reaction volume. The reaction was stopped by heat denaturing samples at 100°C for 5 min. Sample manipulations were internally controlled by adding 100 μL of a galactosamine (GalN) solution to the reaction mixture. Dionex OnGuard II RP cartridges were used to remove the detergent and lipid. The analysis was carried out on a Dionex high-performance anion-exchange chromatography device, coupled with a pulsed amperometric detection apparatus (HPAE-PAD), using a CarboPac PA-10 analytical column equipped with a CarboPac PA-10 guard column. An isocratic flow of 6 mM NaOH at 0.25 mL/min for 25 min was used to separate monosaccharides (Glc and GalN). The amount of glucose (Glc) was calculated from linear regression analysis of GalN and Glc standards in the range of 10–480 pmol per injection. The assay was carried out in a range of substrate concentrations of 5–150 μM, and obeyed Michaelis-Menten kinetics, thus permitting assignment of *K_m* and *V_{max}* values.

Site-specific characterization of glycans

Velaglycerase alfa (drug substance lot EP06-003, Shire Human Genetic Therapies, Hampshire, UK) and imiglycerase (commercial product lot HA163BL) were prepared for enzymatic digestion by reductive denaturation with DTT, followed by and

cysteine alkylation with iodoacetic acid. Alkylated samples were digested first with the endoproteinase Lys-C (Roche Diagnostics GmbH, Mannheim, Germany) (1:42 enzyme to substrate ratio, w/w, for 6 h at 37°C), followed by digestion with endoproteinase Glu-C (1:25 enzyme to substrate ratio, w/w, for 16 h at room temperature). Digested samples were analyzed by peptide mass mapping using reversed phase chromatography with in-line UV (214 nm) and electrospray ionization with mass spectrometric detection (LC-ESI-MS). By comparing the peptide maps before and after glycan release using *N*-glycosidase F (New England Biolabs, Ipswich, MA), the five potential glycosylation sites were identified. The glycan mass was calculated by subtracting the expected peptide mass from the observed glycopeptide masses. Using software to match the observed glycan masses with potential monosaccharide compositions, glycan compositions for each site were determined. To verify monosaccharide compositions, treatments (according to manufacturer's recommendations) with neuraminidase (Roche Diagnostics GmbH), alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany), and α -mannosidase (Glyko, Inc., Hayward, CA) were used to verify the presence of sialic acid, phosphate, and alpha-linked mannose, respectively. MS/MS fragmentation analysis was used to verify glycan phosphorylation.

Glycan map analysis

The procedure involves heat denaturation of the protein at 100°C for 3–4 min in the presence of 0.5% SDS, followed by enzymatic release of glycans with *N*-glycosidase F (Prozyme, San Leandro, CA). Velaglycerase alfa (drug substance lot EP06-001, Shire Human Genetic Therapies) was incubated with *N*-glycosidase F (30 mU/3 μ L) for 4–6 h at 37°C with 0.9% NP40, followed by a second addition of *N*-glycosidase F, and an additional 17–19 h incubation at 37°C. Analysis of the released glycans was performed by HPAE-PAD, using a CarboPac PA-1 analytical column equipped with a CarboPac PA-1 guard column (Dionex, Sunnyvale, CA). Glycans were applied to the column in 12 mM sodium acetate/100 mM NaOH, followed by elution with a 12–300 mM sodium acetate gradient (6.4 mM/min) in 100 mM NaOH in 45 min. Using a flow rate of 1 mL/min and the column at ambient room temperature, glycans elute in the order of increasing negative charge.

Cellular internalization

Human U937 cells were cultured in growth media containing RPMI 1640 with 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, and 10% FBS. Treatment with phorbol myristate acetate (PMA) for 3 days was used to induce differentiation into macrophages (Amento et al. 1984). The U937-derived macrophages were seeded into 96-well microtiter plates at 50,000 cells per well in growth medium, and allowed to adhere to the plates for 48 h. Seeded macrophages were incubated for 3 h with equimolar preparations of velaglycerase alfa (drug substance lot FEC06-003, Shire Human Genetic Therapies) or imiglucerase (Cerezyme®; commercial product lot C7036C01, Genzyme, Cambridge, MA) at pH 7.5, in growth medium containing RPMI 1640 devoid of phosphate, 0.1% BSA, 10 mM HEPES, pH 7.5, 2 mM L-glutamine, 1 mM DTT, and 10 mM CaCl₂. In all assays, the cells were treated with GlcCer for a 3-h duration which was previously determined to be in the

linear range of internalization. For dose response curves utilized to demonstrate mannose-receptor specificity, 10 mg/mL mannan was used to antagonize the receptor. After a series of wash steps (wash buffer: 0.05 M Tris, 0.138 M NaCl, 0.0027 M KCl, with 0.05% Tween 20, 0.5% BSA, pH 8.0), the cells were lysed (lysis buffer: 10 mM Tris pH 8.0, 0.5% NP40, 0.2% deoxycholate, Complete Mini Protease Inhibitor Cocktail Tablets in EASYpacks and PhosSTOP Phosphatase Inhibitor Cocktail Tablets in EASYpacks, Roche Applied Science), and the internalized GlcCer was quantified by an assay employing the synthetic substrate, 4-methylumbelliferyl- β -D-glucopyranoside (4-MU-glc), which releases a fluorescent product upon cleavage. The protein content in the well was determined (BCA method according to the manufacturer's protocol) and was used to normalize the assay signal to total protein from each sample. The assay signal for the GlcCer samples was tested in vitro to determine the extent of activity or signal disparity between the two drugs, and there was no difference in activity (data not shown). For these assays, 2-fold serial dilutions of velaglycerase alfa and imiglucerase (starting at 30 nM enzyme) were made in the assay lysis buffer and tested using the 4-MU-glc enzymatic activity assay. Plates were read with a Perkin Elmer Envision multi-label plate reader.

Funding

Shire Human Genetic Therapies, Inc.

Acknowledgements

J.L. Sussman is the Morton and Gladys Pickman Professor of Structural Biology, and A.H. Futerman is the Joseph Meyerhoff Professor of Biochemistry at the Weizmann Institute of Science. The contribution of Meng Wu, for technical assistance is gratefully acknowledged. We are grateful to Dr. Hilary Voet (Faculty of Agriculture, The Hebrew University, Rehovot) for invaluable discussions concerning the statistical analysis of the choice of space groups.

Conflict of interest statement

None declared.

Abbreviations

CHO, Chinese hamster ovary; ERT, enzyme replacement therapy; GA-GCB, velaglycerase alfa; GlcCer, glucosylceramide; GlcCerase, acid- β -glucosidase; M6P, mannose-6-phosphate; prGCD, GlcCerase expressed in transgenic carrot cells; RMSD, root mean square deviation.

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In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment G

Letter from FDA to Transkaryotic Therapies, Inc., dated January 12, 2004,
providing the IND number and showing the date of receipt by FDA of the IND



DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Rockville, MD 20857

JAN 19 2004

IND 61,220

Transkaryotic Therapies, Inc.
Attn: Suzanne L. Bruhn, Ph.D.
Vice President, Regulatory Affairs
700 Main Street
Cambridge, MA 02139

Dear Dr. Bruhn:

We acknowledge receipt of your Investigational New Drug Application (IND) submitted under section 505(i) of the Federal Food, Drug, and Cosmetic Act. Please note the following identifying data:

IND Number Assigned: 61,220

Sponsor: Transkaryotic Therapies, Inc.

Name of Drug: Gene-Activated® Glucocerebrosidase (GA-GCB, DRX008A)

Date of Submission: December 30, 2003

Date of Receipt: December 31, 2003

Studies in humans may not be initiated until 30 days after the date of receipt shown above. If, on or before January 30, 2004, we identify deficiencies in the IND that require correction before human studies begin or that require restriction of human studies, we will notify you immediately that (1) clinical studies may not be initiated under this IND ("clinical hold") or that (2) certain restrictions apply to clinical studies under this IND ("partial clinical hold"). In the event of such notification, you must not initiate or you must restrict such studies until you have submitted information to correct the deficiencies, and we have notified you that the information you submitted is satisfactory.

It has not been our policy to object to a sponsor, upon receipt of this acknowledgement letter, either obtaining supplies of the investigational drug or shipping it to investigators listed in the IND. However, if the drug is shipped to investigators, they should be reminded that studies may not begin under the IND until 30 days after the IND receipt date or later if the IND is placed on clinical hold.

As sponsor of this IND, you are responsible for compliance with the Federal Food, Drug, and Cosmetic Act and the implementing regulations (Title 21 of the Code of Federal Regulations). Those responsibilities include (1) reporting any unexpected fatal or life-threatening adverse experience associated with use of the drug by telephone or fax no later than 7 calendar days after initial receipt of the information [21 CFR 312.32(c)(2)]; (2) reporting any adverse experience associated with use of the drug that is both serious and unexpected in writing no later than 15 calendar days after initial receipt of the information [21 CFR 312.32(c)(1)]; and (3) submitting annual progress reports [21 CFR 312.33].

Please forward all future communications concerning this IND in triplicate, identified by the above IND number, to the following address:

U.S. Postal Service/Courier/Overnight Mail:
Food and Drug Administration
Center for Drug Evaluation and Research
Division of Metabolic and Endocrine Drug Products, HFD-510
Attention: Fishers Document Room, 8B-45
5600 Fishers Lane
Rockville, Maryland 20857

If you have any questions, call me at (301) 827-6416.

Sincerely,

{See appended electronic signature page}

Patricia Madara
Regulatory Project Manager
Division of Metabolic & Endocrine Drug Products
Office of Drug Evaluation II
Center for Drug Evaluation and Research

**This is a representation of an electronic record that was signed electronically and
this page is the manifestation of the electronic signature.**

/s/

Patricia Madara
1/12/04 02:28:37 PM

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNANOSE PROTEINS AND METHODS OF MAKING HIGH
MANNANOSE PROTEINS

Attachment G1

A written record of the discussion that occurred on January 28, 2004 regarding
modification of the protocol

**FDA CONTACT REPORT
DRX008A**

Contact:	Pat Madara, Metabolic and Endocrinologic Group
	Phone: 301-827-6416
Date:	28 Jan 2004
Time:	10:30

TKT Participants: Steve Schmitz (SS)

Executive Summary:

- Pat Madara telephoned to say that Dr. Pariser, the medical reviewer for the IND submission, informed her that she had not received the protocol amendment, which incorporated the changes discussed at the Pre-IND meeting.
- If she did not receive the amendment, the program would be placed on clinical hold.
- I informed her that we were prepared to send out the amendment within the next 1-2 days.

Action Item:

- Send out the protocol amendment to Pat Madara.

Signature: 

Date: 28 Jan 2004

Copy List: W. Aliski
R. Fram

RA Archives (original)
RA Chronology

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment G2

Letter from Transkaryotic Therapies, Inc. to FDA dated March 11, 2004,
concerning amendment of protocol

11 March 2004

Patricia Madara
Regulatory Project Manager
Division of Metabolic and Endocrine Drug Products
Office of Drug Evaluation II
Center for Drug Evaluation and Research
5600 Fishers Lane
Rockville, MD 20857

RE: IND# 61,220
Amendment 2.0, Clinical Protocol TKT025
Serial No. 002

Product Name: Mannose-terminated, Gene-Activated® Glucocerebrosidase,
(GA-GCB, DRX008A)

Dear Ms. Madara:

Please find enclosed a copy of Amendment 2.0 to Clinical Protocol TKT025 (Attachment 1), and a document entitled, "Listing of Changes in Amendment No. 2 to TKT Clinical Protocol No. TKT025 (Attachment 2).

The changes include:

- 1) Addition of a MRI of the lumbar spine. Originally, the MRI evaluation examined only the femora and abdomen. However, in order to enhance the assessment of bone marrow involvement by the Bone Marrow Burden Score (see below), an MRI of the lumbar spine is required.
- 2) The addition of an exploratory clinical activity variable, the bone marrow burden score. This score is obtained by evaluating MRI images of both the axial (lumbar spine) and peripheral (femora).
- 3) Clarification of timing of vital sign determination
- 4) Itemization, by "bulleting", in the Schedule of Events, to specify electrocardiogram testing at Weeks 21 and 33.
- 5) Updating of the Informed Consent to describe the additional tests mentioned above. In addition, a statement regarding the potential eligibility of a patient who declined to enter the study, to receive approved therapy (i.e., imiglucerase), was deleted. TKT believed that the statement, as written in the Amendment 1.0, had potential to be misleading to patients. We were concerned that a patient could possibly interpret the previous wording to mean that, in the event that he declined to participate in the study, the Sponsor would provide imiglucerase, a currently approved therapy for Gaucher disease.

If you have any questions or comments, please telephone me at 617-613-4364. Thank you for your consideration.

Sincerely,

A handwritten signature in black ink, appearing to read "Stephen M. Schmitz". The signature is fluid and cursive, with the first name "Stephen" and last name "Schmitz" clearly distinguishable.

Stephen M. Schmitz, M.D., M.P.H.
Director, Safety and Regulatory Affairs
Transkaryotic Therapies, Inc.

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment G3

FDA communication to Transkaryotic Therapies, Inc. dated May 20, 2004,
concerning amendment to protocol

AGENCY CORRESPONDENCE

GCB

Contact(s)	Pat Madara, Project Manager Div. of Metabolic and Endocrinologic Drug Products/CDER/FDA 301-827-6416
Date	20 May 2004
Time	1400 and 1415 (Hours and Minutes in Military Time)

Agency Participants

Pat Madara, Project Manager (PM)

Re: IND 61,220 – DRX008A (glucocerebrosidase, GA-GCB)

TKT Participants

Suzanne L. Bruhn, VP, Reg. Affairs (SB)

Alyssa Sonntag, Project Manager, Reg. Affairs (AS)

Executive Summary

- FDA has no comments on the blinding procedure to be implemented for the analysis of liver and spleen volumes from MRI scans taken for Study TKT025. TKT will make no changes to the current version of the blinding procedure (dated 7 April 2004).

Summary

At 1400, SB called PM to inquire on the status of FDA's review of the blinding procedure for analysis of liver and spleen volumes from the MRI scans taken for Study TKT025, which was submitted to the IND in Serial 003 on 7 April 2004. PM stated that the Medical Officer's review of this submission had been completed but she would need to confirm if there were any comments to relay to the sponsor from that review. PM stated that she would contact us soon with this information, but we should call her again after 2 weeks if we had not heard from her. PM will be out of the office during the last week of May and the first week of June.

PM called back at 1415 and informed SB that she had pulled the Medical Officer's review and that it was "safe to proceed [with the blinding procedure] as amended." Therefore, TKT will make no changes to the current version of the blinding procedure (dated 7 April 2004).

Serial Submissions Discussed

Serial 003, submitted to IND 61,220 on 7 April 2004

Action Item(s)

- None

Signature Alysa Fountez **Date** 24 May 2004

Copy List Regulatory Chronology (Original w/ signature)

S. Bruhn

R. Fram

S. Zildjian

N. Wyant

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment H

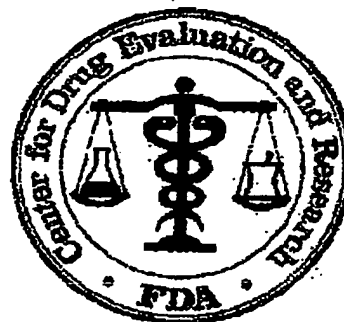
Letter from FDA to Shire Human Genetic Therapies, Inc. indicating the date the
IND was put on clinical hold

REGULATORY AFFAIRS
RECEIVED

NOV 28 2006

FAX

SHIRE HGT



**FOOD AND DRUG ADMINISTRATION
DIVISION OF GASTROENTEROLOGY PRODUCTS**
Center for Drug Evaluation and Research, HFD-180
10903 New Hampshire Ave, Silver Spring, MD 20993-0002

To: Nikhil S. Mehta, Ph.D.

From: Ryan Barraco

Fax: 617-613-4444

Fax: 301-796-9905

Phone: 617-613-4531

Phone: 301-796-0846

Pages, including cover sheet: 8

Date: November 28, 2006

Re: IND 61,220 for GA-GCB – Full Clinical Hold Letter

Comments:

Courtesy Fax

THIS DOCUMENT IS INTENDED ONLY FOR THE USE OF THE PARTY TO WHOM IT IS ADDRESSED AND MAY CONTAIN INFORMATION THAT IS PRIVILEGED, CONFIDENTIAL AND PROTECTED FROM DISCLOSURE UNDER APPLICABLE LAW. If you are not the addressee, or a person authorized to deliver the document to the addressee, you are hereby notified that any review, disclosure, dissemination or other action based on the content of the communication is not authorized. If you have received this document in error, please immediately notify us by telephone and return it to us at the above address by mail. Thank you.

**DEPARTMENT OF HEALTH & HUMAN SERVICES**

Public Health Service

Food and Drug Administration
Rockville, MD 20857

IND 61,220

FULL CLINICAL HOLD

Shire Human Genetic Therapies
Attention: Nikhil Mehta, Ph.D.
Vice President, Global Regulatory Affairs
700 Main Street
Cambridge, MA 02139

Dear Dr. Mehta:

Please refer to your Investigational New Drug Application (IND) submitted December 30, 2003, received December 31, 2003, under section 505(i) of the Federal Food, Drug, and Cosmetic Act for Gene Activated® Glucocerebrosidase (GA-GCB).

We also refer to your amendment dated August 3, 2006 (serial # 035), and to the November 20, 2006, telephone conversation between you and our Division, in which you were notified that your IND is on clinical hold and any proposed studies may not be initiated. The following are the specific deficiencies [21 CFR 312.42(b)] and the information needed to resolve these deficiencies.

Insufficient information to assess risks to human subjects [21 CFR 312.42(b)(2)(ii)].

Clinical Hold Deficiencies

The chemistry, manufacturing, and controls (CMC) amendment dated August 3, 2006, did not demonstrate that the two manufacturing processes for Gene Activated® Glucocerebrosidase (GA-GCB) yield drug substances (DS) with comparable physicochemical characteristics. The comparability data provided in this amendment revealed that there were the following physicochemical differences between DS produced by the two processes:

- a. For the glycan mapping, the total percentage of Group 1 carbohydrates is different between the two DS, and it appears that relative proportions of individual peaks within Group 1 are different in the two DS.
- b. Although the predominant peaks C, D, and E in IEX-HPLC constitute more than 75% of the total peak area and meet the acceptance criteria, there is a significant shift in the proportion of each peak, which does not appear to be due to assay variability.
- c. The pattern of bands detected by IEF gels Coomassie stained is different in DS manufactured with the serum containing process (E303-006) versus DS

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manufactured with the animal-free process (EP06-003). At least one additional acidic species is present in E303-006.

As you intend to use the DS manufactured using the new manufacturing process in your proposed Phase 3 clinical study, and as this DS has not been evaluated in pre-clinical or clinical studies, insufficient information exists with this DS to assess the risks to human subjects for the proposed Phase 3 clinical investigation.

Information needed to resolve clinical hold deficiencies

1. You must demonstrate the comparability of DS by the two manufacturing processes as set forth in the "Guidance for Industry Q5E Comparability of Biotechnological/Biological Products Subject to Changes in their Manufacturing Process."
2. Alternatively, you may propose to perform your Phase 3 clinical study using the same DS administered in the completed Phase 1/2 study and in the pre-clinical testing conducted in support of this Phase 1/2 study.
3. Alternatively, you may propose to repeat pre-clinical and clinical studies with DS manufactured using the new manufacturing method, which are needed to support the proposed Phase 3 clinical study. These studies are to include:
 - a. A head-to-head comparison of the two DS in *in vitro* and *in vivo* pharmacology studies to demonstrate comparability of the two DS on the primary pharmacological effect of GA-GCB.
 - b. A head-to-head tissue distribution comparison of the two DS in Sprague Dawley rats of both sexes.
 - c. A clinical study to assess the pharmacokinetics, pharmacodynamics, and preliminary safety of GA-GCB administration.

Until you have submitted the required information, and we notify you that you may initiate the trial, you may not legally conduct the identified clinical study under this IND.

Please identify your response to the clinical hold issues as a "CLINICAL HOLD COMPLETE RESPONSE." To facilitate a response to your submission, submit this information in triplicate to the IND. In addition, send a copy of the cover letter to Ryan Barraco.

Following receipt of your complete response to these issues, we will notify you of our decision within 30 days.

In addition, we have the following recommendations and requests that are important for product development, but are not clinical hold issues at this time. Your responses to any non-hold issues should be addressed in a separate amendment to the IND.

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CMC

Regarding characterization and release testing:

1. You are currently measuring enzymatic activity using a surrogate substrate. In order to properly characterize DS and drug product (DP), measurements of the K_m and k_{cat} kinetic parameters using a physiologically relevant substrate should be performed.
2. Routine DS and DP release testing should also include (i) measurements of the K_m and k_{cat} kinetic parameters using a physiologically relevant substrate, and (ii) a quantitative assessment of receptor binding and uptake by macrophages. Please note that glycan mapping is not considered to be a potency assay from a regulatory perspective.
3. An in-depth characterization of the glycan structures in GA-GCB, with information on branching and size of the glycan chains, mannose-6P content and residual content of NANA should be performed. Adequate assays that allow for control of carbohydrate content and structure should be included in release testing.
4. The area for each peak identified in groups 1, 2 and 3 of the glycan mapping assay should be specified in your release testing.
5. N-terminal sequencing and Western blotting for identity, and Ion Exchange Chromatography for purity were not performed at release testing for the clinical lots of DS. These tests assess critical product attributes and should be maintained as release tests.
6. Your current DS acceptance criteria for RP-HPLC and SE-HPLC are $\geq 94\%$ of the main peak area. From the results of batch analysis, it appears that on average, RP-HPLC purity is about 98% and SE-HPLC is about 97%. Acceptance criteria should be established based on process capability and manufacturing experience, and we recommend establishing more stringent specifications.
7. It appears that the new process generates a DS with a higher percent of aggregates (by comparison of batch analysis results). These aggregates should be characterized, and an orthogonal method to detect aggregates should be used to validate SE-HPLC.
8. It is not clear whether studies were performed to detect impurities that could arise from the DP manufacturing process. The increase in aggregation of GA-GCB during DP manufacturing should be documented, and procedures implemented to minimize aggregate formation.

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9. You have significantly changed DP acceptance criteria for SE-HPLC and RP-HPLC to $\geq 92\%$ main peak area. Although you justify these acceptance criteria based on limited manufacturing experience, they appear to allow for excessive amounts of aggregates and impurities in the DP. Acceptance criteria should be established based on process capability and manufacturing experience, and we recommend establishing more stringent specifications.

Regarding the manufacturing process:

10. You provided flow charts for the manufacturing process that include in-process controls. It appears that the only in-process control in the purification step is protein recovery. We recommend inclusion of additional in-process tests that could provide information on purity such as, but not limited to, SDS-PAGE reducing and non-reducing.
11. You stated that impurities derived from the culture medium, such as plant hydrolysates, kifunensine and DTT will be removed during the purification process. You should provide supportive data for the above claims. Presence of impurities should be assessed and specified at critical steps, and in lot release. Alternatively, removal of the process-related impurities must be validated.
12. Acceptance criteria for HCP content should be modified to reflect the actual capability of the process to remove these impurities. Currently, acceptance criterion is <200 ng/mg, and the actual results range from 7 to 23 ng/mg.
13. You state that antibodies have been raised against protein lysates from cells growing in serum-free and serum-containing medium. Please clarify which antibodies have been used to develop the ELISA and Western blotting assays.

Regarding stability:

14. It is important to demonstrate that critical drug potency parameters are not altered at the indicated storage temperature. Please include the following assays in your stability testing program for DS and DP:
 - a. Macrophage uptake assay and receptor binding assay.
 - b. Measurements of the K_m and k_{cat} kinetic parameters.
 - c. All assays should be evaluated for stability indicating potential.
15. Please refer to ICH Q5C for guidance on stability studies for biotechnology products.

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Regarding cell banks:

16. You provided a stability program for MCB and WCB of up to four years. Viability and growth should be assessed at later times as well, to ensure that a constant supply of starting material is reproducibly available.
17. Please submit data regarding viral clearance by filtration for review as soon as they are available.

Clinical/Statistical**Regarding your proposed Phase 3 clinical protocol:**

18. Your protocol contains a large number of secondary endpoints. If you intend to use any of these secondary endpoints to support the indication for the treatment of type 1 Gaucher disease with GA-GCB, you will need to include in your statistical analysis plan a proposal for evaluating these endpoints in a statistically rigorous manner that accounts for multiplicity.
19. Please provide a rationale for performing pharmacokinetic sampling to evaluate the multiple-dose pharmacokinetics of GA-GCB at Week 37.
20. Your protocol excludes from study participation patients who are anti-imiglucerase IgG antibody positive. As stated at the End of Phase 2 (EOP2) meeting on January 11, 2006, it is likely that at least some of the patients in clinical practice who transition from Cerezyme® to GA-GCB will be IgG anti-imiglucerase antibody positive. We recommend that the inclusion criteria be broadened to include type 1 Gaucher Disease patients regardless of imiglucerase-antibody status, as inclusion of these patients would more accurately represent the expected clinical use of GA-GCB, and would support the use of GA-GCB in a broader patient population.
21. The stopping rules for your study (in protocol section 9.4) state that "If any patient experiences a life-threatening (Grade 4) serious adverse event (SAE), or death occurs that is considered possibly or probably related to the study drug, the decision to stop the study requires agreement by the Shire HGT Medical Monitor, the Investigator, and the IRB/IEC." Please revise the stopping rules for the study based on specific safety criteria, rather than on the subjective assessment of events by study personnel.
22. The procedures for Serious Adverse Event (SAE) reporting (in protocol section 9.3.1) state that "Any SAE that occurs after administration of the first dose of GA-GCB must be reported in the event of a severe, possibly or probably related AE or SAE..." Please revise the SAE reporting procedures in your protocol to more accurately reflect the requirements under 21 CFR 312.32(c)(1) and (2), whereby "The sponsor shall notify FDA and all participating investigators in a written IND safety report of: (A) Any adverse experience associated with the use of the drug that is both serious and

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unexpected..." and "The sponsor shall also notify FDA by telephone or by facsimile transmission of any unexpected fatal or life-threatening experience associated with the use of the drug... in no event later than 7 calendar days..." Please note that this requirement does not include a subjective assessment (possibly or probably related) of the event.

23. Please revise your protocol so that all pediatric patients participating in the study are to undergo assessments of growth at regular intervals in the study. Assessments of growth including, at minimum, assessments of height and weight, are to be obtained in a standardized manner that are to be delineated in the study protocol (e.g., height measured via a calibrated stadiometer, and the final measurement taken as an average of three measurements).
24. Your sample Informed Consent form states (on page 5, paragraph 7) that children as young as two years of age will be undergoing magnetic resonance imaging (MRI) testing. In young children, sedation is often required for MRI testing. We recommend that you revise your sample Informed Consent form to note the possible need for sedation in pediatric patients for MRI testing, and an explanation of the risks of sedation in these patients.

Please cite the IND number listed above at the top of the first page of any communications concerning this application. Send all submissions, electronic or paper, including those sent by overnight mail or courier, to the following address:

Food and Drug Administration
Center for Drug Evaluation and Research
Division of Gastroenterology
5901-B Ammendale Road
Beltsville, MD 20705-1266

If you have any questions, call Ryan Barraco, Regulatory Project Manager, at (301) 796-0846.

Sincerely,

{See appended electronic signature page}

Brian E. Harvey, M.D., Ph.D.
Director
Division of Gastroenterology Products
Office of Drug Evaluation III
Center for Drug Evaluation and Research

**This is a representation of an electronic record that was signed electronically and
this page is the manifestation of the electronic signature.**

/s/

Brian Harvey
11/27/2006 04:00:35 PM

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment I

Letter from FDA to Shire Human Genetic Therapies, Inc., dated December 21,
2006, removing the clinical hold and indicating that the protocol can be initiated



DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Rockville, MD 20857

IND 61,220

Shire Human Genetic Therapies
Attention: Nikhil Mehta, Ph.D.
Vice President, Global Regulatory Affairs
700 Main Street
Cambridge, MA 02139

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Dear Dr. Mehta:

Please refer to your Investigational New Drug Application (IND) submitted December 30, 2003, under section 505(i) of the Federal Food, Drug, and Cosmetic Act for Gene Activated Glucocerebrosidase (GA-GCB).

We also refer to your amendment dated November 30, 2006 (serial # 041), which provided a complete response to our December 7, 2006, letter which cited the reasons for placing Protocol TKT032, titled "A Multi-center, Randomized, Double-Blind, Parallel Group, Two-Dose Study of Gene-Activated™ Human Glucocerebrosidase (GAGCB) Enzyme Replacement Therapy in Patients with Type I Gaucher Disease," on clinical hold and the information needed to resolve the clinical hold issues.

We have completed the review of your submission, and have concluded that the above protocol may be initiated.

We have the following comments, however, regarding your clinical development program:

A direct comparison of IEX-HPLC and glycan mapping data for drug substance (DS) lots E303-005, E303-006, E303-007, and EP06-003 indicate that physico-chemical differences exist between the DS manufactured with different processes that were used in clinical trials. However, these differences do not appear to pose a serious safety risk to human subjects, and clinical trials with the Animal Free (AF) DS appear to be safe to proceed at this time. Nevertheless, in view of these differences, please be aware that you might not be able to use the Phase 1 clinical data generated using the serum-containing DS to support a future marketing application for GA-GCB.

As sponsor of this IND, you are responsible for compliance with the Federal Food, Drug, and Cosmetic Act and the implementing regulations (Title 21 of the Code of Federal Regulations). Those responsibilities include (1) reporting any unexpected fatal or life-threatening adverse experience associated with use of the drug by telephone or fax no later than 7 calendar days after initial receipt of the information [21 CFR 312.32(c)(2)]; (2) reporting any adverse experience associated with use of the drug that is both serious and unexpected in writing no later than 15 calendar days after initial receipt of the information [21 CFR 312.32(c)(1)]; and (3) submitting annual progress reports [21 CFR 312.33].

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If you have any questions, call Ryan Barraco, Regulatory Project Manager, at (301) 796-0846.

Sincerely,

{See appended electronic signature page}

Brian E. Harvey, M.D., Ph.D.
Director
Division of Gastroenterology Products
Office of Drug Evaluation III
Center for Drug Evaluation and Research

REGULATORY AFFAIRS
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JUN 5 1997

SHIRE HGT

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/s/

Brian Harvey
12/21/2006 05:11:30 PM

In re U.S. Patent No.: 7,138,262 B1

Attorney Docket No.: S2071-701019/0013US

Issued: November 21, 2006

Inventors: Peter Francis Daniel

Assignee: Shire Human Genetic Therapies,
Inc.

Title: HIGH MANNOSE PROTEINS AND METHODS OF MAKING HIGH
MANNOSE PROTEINS

Attachment J

Letter from FDA to Shire Human Genetic Therapies, Inc., dated September 14,
2009, acknowledging receipt of the final submission of the NDA



DEPARTMENT OF HEALTH AND HUMAN SERVICES

Food and Drug Administration
Silver Spring MD 20993

NDA 22575

Shire Human Genetic Therapies, Inc.
Attention: Nikhil Mehta, Ph.D.
Vice President, Global Regulatory Affairs
700 Main Street
Cambridge, MA 02139

NDA ACKNOWLEDGMENT



Dear Dr. Mehta:

We have received your new drug application (NDA) submitted under section 505(b) of the Federal Food, Drug, and Cosmetic Act (FDCA) for the following:

Name of Drug Product: TRADENAME (velaglucerase alfa)

Date of Application: August 31, 2009

Date of Receipt: August 31, 2009

Our Reference Number: NDA 22575

Unless we notify you within 60 days of the receipt date that the application is not sufficiently complete to permit a substantive review, we will file the application on October 30, 2009, in accordance with 21 CFR 314.101(a).

If you have not already done so, promptly submit the content of labeling [21 CFR 314.50(l)(1)(i)] in structured product labeling (SPL) format as described at <http://www.fda.gov/oc/datacouncil/spl.html>. Failure to submit the content of labeling in SPL format may result in a refusal-to-file action under 21 CFR 314.101(d)(3). The content of labeling must conform to the content and format requirements of revised 21 CFR 201.56-57.

The NDA number provided above should be cited at the top of the first page of all submissions to this application. Send all submissions, electronic or paper, including those sent by overnight mail or courier, to the following address:

Food and Drug Administration
Center for Drug Evaluation and Research
Division of Gastroenterology Products
5901-B Ammendale Road
Beltsville, MD 20705-1266

All regulatory documents submitted in paper should be three-hole punched on the left side of the page and bound. The left margin should be at least three-fourths of an inch to assure text is not obscured in the fastened area. Standard paper size (8-1/2 by 11 inches) should be used; however, it may occasionally be necessary to use individual pages larger than standard paper size. Non-standard, large pages should be folded and mounted to allow the page to be opened for review without disassembling the jacket and refolded without damage when the volume is shelved. Shipping unbound documents may result in the loss of portions of the submission or an unnecessary delay in processing which could have an adverse impact on the review of the submission. For additional information, please see <http://www.fda.gov/cder/ddms/binders.htm>.

If you have any questions, call me at (301) 796-0069.

Sincerely,

{See appended electronic signature page}

R. Wesley Ishihara
Regulatory Health Project Manager
Division of Gastroenterology Products
Office of Drug Evaluation III
Center for Drug Evaluation and Research

Application
Type/Number

Submission
Type/Number

Submitter Name

Product Name

NDA-22575

ORIG-1

SHIRE HGT INC.

VELAGLUCERASE ALFA

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/s/

RICHARD W ISHIHARA
09/14/2009